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THE INFLUENCE OF EPINEPHRINE AND INSULIN ON THE DISTRIBUTION OF GLYCOGEN IN RABBITS.*

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I. *Effect of Epinephrine on Glycogen Metabolism.*

As a background for a study of the effect of epinephrine on insulin action we have found it necessary to inquire into the changes in glycogen distribution which follow upon the administration of epinephrine. We considered it especially important to understand the relationship which holds between the time of action of the hormone and the quantitative changes in glycogen distribution. This time factor, although practically ignored by most investigators, soon proved to be of major importance. Failure to give it adequate consideration accounts, in our opinion, for much of the disagreement revealed in the literature on glycogen metabolism. Of nineteen papers which we examined, ten necessitated the conclusion that epinephrine decreases the concentration of liver glycogen and seven demonstrated *per contra* that epinephrine favors the formation of liver glycogen. In this paper we propose to show that both of these concepts, opposing as they may appear to be, are experimentally demonstrable and valid. Both views can be reconciled if due attention is given to the time during which the epinephrine is permitted to act. There is an initial period during which hyperglycemia develops and both hepatic and muscle glycogen decrease. There is a later period during which the decrease in muscle glycogen continues but in which the liver not only regains its normal glycogen content but proceeds to deposit enormous quantities of the polysaccharide.

* The material in this paper is drawn from the thesis submitted by Melville Sahyun as a candidate for the degree of Master of Arts at Stanford University.

We were also drawn to consider a second factor, the age of the experimental animals. Failure on the part of many investigators to control this variable has contributed to some of the confusion. In view of the marked relationship in rabbits between age and insulin resistance (1) we were inclined to suspect that a similar correlation might exist between age and changes in glycogen distribution in response to epinephrine or insulin. In this we have been anticipated by Goldblatt (2) who worked with very young rabbits.

Experimental Animals.

The animals used were young adult rabbits of 1.8 to 2.2 kilos at the time of experiment, all having been fasted for 24 hours before use unless specified otherwise in the tabulated data. All were of a single variety (Belgian browns). They were obtained from a single source and were maintained upon a uniform diet of alfalfa and barley. The alfalfa hay was given daily and barley twice a week. The experiments were confined to the winter and early spring months.

We consider the exercise of scrupulous care in the selection and maintenance of animals to be of major importance. There is no doubt that great variations in the age, and nutritional conditions of the experimental animals have contributed much to the uncertainty and confusion of some of the early data. Evidence is also on record that the glycogen content of animal tissues suffers a seasonal variation (3, 4) and that the response of animals to insulin is likewise subject to seasonal changes (1).

Other factors which, by receiving inadequate attention, have led to uncertain and erroneous interpretations should be enumerated. They are six or seven in number: (1) faulty analytical methods employed in the determination of glycogen, (2) delays in commencing the glycogen determinations with resulting glycolysis, (3) too few experiments, (4) the omission of controls, (5) wide variations in the size and age of animals, (6) the use of different animal species, and (7) differences in the nutritional state of the animals at the time of experiment.

For the experiments reported in Table IV large rabbits of 3.5 to 4.0 kilos were necessarily employed.

Experimental Technique.

As recommended by Simpson and Macleod (5), scrupulous care was taken to minimize postmortem glycogenolysis. The animals were killed by stunning and the tissue samples rapidly excised, weighed, and immersed in hot potassium hydroxide. Through an abdominal incision the liver was first removed, *in toto*. The right (or left) sciatic nerve was then severed. A 25 gm. sample of liver was weighed out, cut into three or four pieces, and dropped into a flask containing 25 cc. of hot 60 per cent potassium hydroxide. Meanwhile the flask was suspended in a bath of boiling water. With experience, this part of the procedure required little more than 1 minute. The right (or left) sartorius muscle was then excised and weighed. In rabbits of about 2 kilos it was found to approximate 25 gm. The muscle was cut longitudinally into three or four strips and dropped into a second flask containing 25 cc. of hot potassium hydroxide.

It is worth noting that sectioning of the sciatic nerve is of considerable importance. The increase in muscle tone and contraction in the stunned animal is very marked. The denervated limb, however, remains relaxed and motionless and suffers no apparent loss in glycogen during the time in which attention is confined to the liver sample. The longitudinal cutting of the muscle is also important for by so doing little injury is done to the muscle fibers and glycogenolysis is retarded.

In the 100 or so experiments performed in this manner, one person was able to excise the tissues, weigh, and drop them into the hot alkali within 2 minutes. When assistance was available, the time was reduced to about 90 seconds.

For the isolation and hydrolysis of the glycogen Pfüger's method (6) was used. The sugar in the hydrolysate was determined by the method of Folin and Wu (7).

Together with the unknown, three standards of 0.05, 0.1, and 0.2 mg. per 1 cc. were run. This method was quite satisfactory, provided the colorimetric readings fell between 15 and 28 when the standard was set at 20. Usually these three standards were found adequate, but repeating the estimation and using a different dilution of the unknown were quite often necessary.

Blood sugar determinations were made on venous samples by the method of Folin and Wu.

Results.

Experiment 1.—To insure accuracy in technique it was considered advisable to compare the procedure described above with the liquid air technique recommended by Simpson and Macleod (5). Two animals, suspected to differ widely in glycogen content, were selected for the comparison. One was a well fed rabbit; the other had been fasted for 24 hours, then injected with epinephrine and histamine, and killed 18 hours later. On excision of the liver, the first portion was promptly frozen with liquid air,

TABLE I.
Use and Omission of Liquid Air in Glycogen Determinations.

Rabbit No.	Weight.	Tissue.	Glycogen.	
			Liquid air used.	Liquid air not used.
	<i>kg.</i>		<i>per cent</i>	<i>per cent</i>
101	2.2	Liver.	10.5	11.3
		Muscle.	0.77	0.75
102	2.0	Liver.	3.38	3.50
		Muscle.	0.056	0.056

weighed, and dropped into boiling potassium hydroxide. The second portion was treated according to the technique described in a preceding paragraph. For the determination of muscle glycogen the two sartorii were used. The first received the liquid air treatment; the second was not frozen. The results are presented in Table I.

It is apparent that under the conditions of these experiments no advantage attends the use of liquid air.

Experiment 2.—Determinations were made upon four animals after a 24 hour fast, and upon three which had fasted 48 hours. No epinephrine was administered. The results are presented in Table II. For comparative purposes glycogen determinations made on a well fed rabbit are included.

Experiment 3.—Epinephrine was administered¹ after 24 hours

¹ 1 cc. of adrenalin chloride (1 mg.), Parke, Davis and Company, was given. In all the experiments reported in this paper subcutaneous administration was employed.

fasting. The results are presented in Table III. It is to be observed (*cf.* Table II) that both the liver and muscle suffer a rapid and marked loss of glycogen. In 1.5 hours the glycogen content of the liver reaches a minimum and thereafter increases steadily to about the 18th hour. At this time the value is 10- to 20-fold that of the fasting control animals. Thereafter there is a rapid decrease in hepatic glycogen which now approaches normal values. Muscle glycogen remains at a low and variable level. A further decrease is apparent at about the 24th hour at the time of rapid glycogen disappearance from the liver.

The early fall, at least in hepatic glycogen, is probably due to hyperglycemia and accelerated glycogenolysis in the liver, and

TABLE II.
Control Rabbits. No Epinephrine.

Rabbit No.	Blood sugar.	Liver glycogen.	Muscle glycogen.	Remarks.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
11	0.100	0.47	0.62	24 hr. fast.
12	0.103	0.34	0.50	24 " "
21	0.105	0.38	0.40	24 " "
22	0.100	0.50	0.47	24 " "
38	0.093	0.100	0.153	48 " "
39	0.090	0.080	0.155	48 " "
40	0.095	0.31	0.185	48 " "
101		11.3	0.75	Not fasted.

lends further support to the theory that blood sugar originates directly from liver glycogen. Nevertheless, it is to be noted that restoration of the latter sets in long before the blood sugar content decreases to normal, which indicates that the reverse of this hypothesis is less acceptable.

Experiment 4.—To determine the influence of animal size upon the results observed in the preceding experiments determinations were made upon several large rabbits (Table IV). By comparison with Table II, it is to be noted that fasting of the smaller animals reduces the liver glycogen content to a lower value than in the larger animals. In view of the greater metabolic rate of the small animal, per unit of body weight, this relative increase in glycogen loss is to be expected. It should also be observed by comparison

Glycogen Distribution in Rabbits

TABLE III.
Glycogen Content after Injection of 1 Mg. of Epinephrine.

Rabbit No.	Blood sugar.				Glycogen.		Time of killing, after epinephrine.
	Time after epinephrine.				Liver.	Muscle.	
	0	1 hr.	1½ hrs.	2 hrs.			
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>hrs.</i>
16	0.105	0.200			0.093	0.055	1
17	0.111	0.288			0.110	0.057	1
13	0.098		0.308		0.065	0.033	1½
14	0.105		0.308		0.077	0.045	1½
15	0.100		Lost.		0.079	0.030	1½
1	0.080			0.265	0.143	0.080	2
2	0.103			0.335	0.345	0.100	2
24	0.095			0.320	0.255	0.063	2
27	0.100			0.275	0.315	0.054	2
		3 hrs.	4 hrs.	5 hrs.			
5	0.105	0.235			0.90	0.037	3
6	0.091	0.220			1.03	0.062	3
8*	0.091	0.285			0.40	0.050	3
25	0.100	0.335			0.44	0.036	3
9	0.103		0.345		0.80	0.047	4
10	0.104		0.392		0.70	0.069	4
23	0.105		0.320		0.58	0.056	4
26	0.103		0.470		1.12	0.052	4
18	0.111			0.250	1.63	0.026	5
19†	0.103				0.47	0.027	5
20	0.110			0.180	2.29	0.030	5
28	0.118			0.410	0.85	0.036	5
	17 hrs.	18 hrs.	22 hrs.	24 hrs.			
29	0.105				2.34	0.050	17
30	0.100				1.65	0.069	17
31		0.098			3.89	0.030	18
32		0.105			3.73	0.031	18
102‡					3.50	0.056	18
33§			0.100		0.302	0.045	22
35§				0.100	0.265	0.023	24
36§				0.100	0.900	0.024	24
37§				0.098	0.465	0.026	24
121					0.44	0.035	42
122					0.45	0.085	42
123					0.41	0.077	42

* Rabbit 8 weighed 1.6 kilos. All others weighed between 1.8 and 2.2 kilos.

TABLE III—*Concluded*

† Rabbit 19, for some unknown reason, went into convulsions. The low liver glycogen supports the hypothesis that similar low values observed after insulin convulsions are secondary to the vigorous muscle contractions and not to the direct influence of the hormone (*cf.* also Pogany (8), Dudley and Marrian (9)). Nevertheless the hypothesis is discounted by the observations of Goldblatt (2), and Britton, Geiling, and Calvery (10).

‡ Rabbit 102 was very sensitive to epinephrine. It became weak after the injection and was given 0.2 mg. of histamine. Recovery was rapid.

§ In animals killed 22 to 24 hours after epinephrine rigor mortis was observed to set in very rapidly, usually within 5 minutes.

with Table III that the effect of epinephrine on the hepatic glycogen of the large animal is quantitatively quite different from that in the small. On the contrary, both in the fasting controls and epinephrine-treated animals, the muscle glycogen changes appear to be less dependent upon the size of the animal. On the whole, the results indicate the necessity of employing animals of constant size in the study of glycogen metabolism.

TABLE IV.
Glycogen Distribution in Large Rabbits.

Rabbit No.	Weight.	Blood sugar.	Liver glycogen.	Muscle glycogen.	Nutritional condition.
	<i>kg.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
86	4.4		17.5	0.57	Not fasted.
87	3.8		11.3	0.52	" "
78	3.4	0.105	0.65	0.42	Fasted 24 hrs.
79	3.2	0.103	0.62	0.35	" 24 "
75	3.5	0.100	2.06	0.026	" 24 " ; then given 1 mg. of epinephrine and killed 24 hrs. later.
76	3.7	0.095	2.18	0.028	
77	3.5	0.098	2.18	0.045	

Discussion of Experiments with Epinephrine.

It has been freely stated throughout the literature of biochemistry that epinephrine hyperglycemia is associated with a general depletion of tissue glycogen. Cori and Cori (11) in a brief critical review of the subject were able to show that much of the work cited in support of the hypothesis was of questionable value. Faulty analytical methods had been employed in the early investigations and the danger of postmortem glycogenolysis had not been appreciated. While agreeing with the prevailing

view that muscle glycogen was rapidly exhausted by epinephrine, they showed that under the conditions of their own experiments hepatic glycogen actually increased. But even though allowance be made for the technical difficulties mentioned by Cori and Cori it seems to us probable that a residuum of demonstrable fact remains in support of the prevalent hypothesis that epinephrine induces a general exhaustion of glycogen. In our own experiments on rabbits (Table III) we found this to be true during the first 2 or 2½ hours of epinephrine action.² By reference to the literature it appears that a number of others who have contended that epinephrine decreases liver glycogen made their analyses within 2 hours or so of injecting the hormone. Thus Drummond and Paton (13), using small rabbits and large doses of epinephrine, killed the animals within 1½ to 2 hours. Doyon and Kareff (14) injected epinephrine in the portal vein and analyzed the liver 30 minutes later. Wolownik (15) made his determinations 3 hours after injecting the hormone, Cori, Cori, and Pucher (16) 10 to 90 minutes after, and Eadie (17) 1½ hours. In observations made by Olmsted and Coulthard (18) upon the decapitated cat over a period of 2 hours, epinephrine also induced a decrease in liver glycogen. Among the others who reported glycogenolysis following epinephrine might be mentioned Agadschanianz (19) and Gatin-Gruzewska (20). The experiments of the former, although frequently cited, are rendered unacceptable through unsatisfactory technique. Both investigators killed the animals 36 to 40 hours after epinephrine. Bierry and Gatin-Gruzewska (21) reported that epinephrine decreased hepatic glycogen but did not state the time of action of the hormone.³

² We considered the effect of epinephrine dosage on the glycogen changes observed in this initial period by injecting two fasted rabbits with only 0.1 mg. of epinephrine per kilo. The animals were killed 1½ hours later. The analytical values obtained were blood sugar 216 mg. per cent in the first animal and 210 mg. per cent in the second, liver glycogen 0.071 and 0.21 per cent respectively, muscle glycogen 0.075 and 0.041 per cent. There was no glycosuria and the reduction of liver glycogen is quite evident. Cori and Cori (12) have discussed the physiological significance of results which follow the subcutaneous injection of large doses of epinephrine. Under the conditions of our experiments it seems likely that less than 0.0005 mg. of epinephrine was absorbed per minute.

³ It is of no little interest that Blum (22), who first described the glycosuric power of epinephrine, stated that glycosuria developed even in animals

On the other hand, there are several investigations culminating in the comprehensive and masterly work of Cori and Cori which demonstrate that epinephrine increases the hepatic store of glycogen. Among these might be mentioned the contribution of Pollak (23), and that of Kuriyama (24) who administered the hormone daily to fasting rabbits during an 8 to 10 day period and killed them within 7 to 24 hours of the last injection. On the basis of histological evidence Loeper and Crouzon (25) came to a similar conclusion. Finally, Cori and Cori in a number of contributions have reported that 3 hours after injecting the hormone into standard rats the liver glycogen was found to be increased well over the level observed in control animals. This was found to be so in the postabsorptive state (11), in the fasting animal (26), during the absorption of glucose (12) and in the adrenalectomized rat (27). The recent work of Blatherwick and Sahyun (28) partly confirms that of Cori and Cori. If one bears in mind the importance of the time factor it may not be without significance that, when epinephrine and glucose were given simultaneously, the hormone had no effect in promoting the deposition of liver glycogen when permitted to act for only 1 hour (12). This can be readily understood in the light of the results reported in this paper, which serve also to explain the earlier findings of Cori, Cori, and Pucher (16).

As for skeletal muscle, there appears to be almost complete agreement among all investigators that epinephrine reduces markedly its glycogen content. In our own work we have found that this decrease is rapidly induced and long continued. In fasting rabbits recovery does not set in before the 2nd day at least.

Returning to a consideration of the glycogen changes in the liver, we feel somewhat at a loss to explain the 2-fold effect observed.⁴ It seems to us likely that subcutaneously injected

which, by long fasting, had been deprived of all liver glycogen. The statement presumably rests upon unpublished data or is supported by papers to which Blum makes no reference. In the light of modern work it is improbable that fasting alone would cause a disappearance of liver glycogen.

⁴ We have been interested to learn that Lawaczek (29) has observed a parallel change in the hexosephosphate content of the blood. Epinephrine

epinephrine would suffer a fate analogous to that of subcutaneously injected amino acids which show an early and relatively great accumulation in the liver, followed by a rather sharp decrease and a slow but steady accumulation in muscle (30). The analogy must of course be qualified by taking into consideration the small amount of the hormone, the local vasoconstriction which inhibits the absorption of epinephrine from subcutaneous areas, and the rapid destruction which epinephrine is alleged to undergo in the tissues. We are however inclined to supplement the theory proposed by Cori and Cori which emphasizes the peripheral, extrahepatic action of epinephrine by considering an early period in which the hepatic function of the hormone is quite pronounced. We suggest accordingly that the initial period of epinephrine action under the conditions of our experiments is characterized by a relatively great accumulation of epinephrine in the liver and a resultant acceleration in glycogenolysis. This effect, associated with the inhibition in peripheral utilization of blood sugar, as suggested by Cori and Cori, accounts for the hyperglycemia. Within 2 hours the migration or disappearance of epinephrine from the liver sets in and the initial effect of accelerating hepatic glycogenolysis passes away. The peripheral effect of epinephrine persists as long as epinephrine continues to be absorbed and may even increase over a number of hours until absorption is overtaken by destruction of the hormone. As soon as the rate of hydrolysis of hepatic glycogen falls below the rate of synthesis, liver glycogen begins to accumulate. Hyperglycemia persists as long as the loss of blood sugar (principally by peripheral utilization) continues below its genesis in the liver. Finally the peripheral effect of epinephrine decreases (though muscle glycogen may still be low), and the rate of utilization of blood sugar is increased. In consequence of the reduced migration of lactic acid to the liver and the simultaneous increase in the utilization of blood sugar in muscle, hepatic glycogenolysis becomes dominant over glycogen synthesis and a precipitate fall in liver glycogen ensues. Thus there is to be observed, as demonstrated in these experiments, an initial

first causes a decrease of 20 to 30 per cent in blood hexosephosphate, followed within an hour or two by an increase of 10 to 30 per cent above the preepinephrine value. Insulin, on the contrary, causes an initial increase.

fall in liver glycogen (first 2 hours), a subsequent and prolonged increase (to the 18th hour), and finally a rapid decrease. With respect to the physiology of epinephrine we consider that the hormone participates in the control of carbohydrate metabolism both by hepatic and extrahepatic functions. The theory of Cori and Cori emphasizes the latter but in so doing describes the changes in liver glycogen and blood sugar principally as secondary phenomena. We consider it necessary to supplement the theory by attributing to epinephrine the added function of accelerating hepatic glycogenolysis. This is a partial return to the older theories.

II. Effect of Epinephrine and Insulin on Glycogen Metabolism.

After 6 to 7 years of work on the effect of insulin on glycogen metabolism in the normal animal, there is still little agreement among investigators. Dudley and Marrian (9) in 1923 reported that insulin administered to well nourished animals caused a marked reduction in the glycogen content of the liver and skeletal muscle, a conclusion supported by the observations of Barbour, Chaikoff, Macleod, and Orr (31) on hepatic glycogen. Likewise in the fasting animal there is other good evidence that insulin reduced the glycogen content of tissues (31, 32).

McCormick and Macleod (33) on the contrary, concluded from similar experiments of their own that moderate doses of insulin in the normal well fed animal caused no significant change in glycogen distribution. This conclusion was supported and confirmed by the observation of Grevenstuck and Laqueur (34) and others (35). In the fasting rabbit Cori (36) observed no effect on the glycogen distribution (or possibly a slight decrease in liver and total glycogen).

Frank, Hartmann, and Nothmann (37) reported an increase in the glycogen content of liver and skeletal muscle of the well fed animal, due to insulin injections. In this they are in agreement with several others who using the fasted animal have reported likewise (38). The recent work of Goldblatt (2) suggests that even though convulsions ensue insulin continues to promote the deposition of hepatic glycogen in young rabbits.

There can be no doubt that in some instances the range of variation in the glycogen content of so called normal animals

has been so broad as to mask whatever insulin effect may have been brought about (39). Under such conditions an investigator would be compelled to conclude that there was no demonstrable effect or else to rest his alleged positive findings on a very doubtful basis. It is apparent also that some work has been vitiated by insulin overdosage with attendant convulsions or coma.

To avoid the former difficulty resort has been had to a number of profitable devices. The first consisted in the use of the depancreatized animal in which as is well known there is profound hyperglycemia and glycogen depletion (40, 41). Banting, Best, Collip, Hepburn, Macleod, and Noble (42), using this technique, were the first to demonstrate that insulin given to sugar-fed diabetic animals caused a storage of glycogen in the liver, even up to 13 per cent. It appeared likely that the skeletal muscle glycogen was also increased. In view of the confirmation accorded to these findings by various workers (36, 43), it is now established with certainty that insulin restores to the diabetic animal the capacity for synthesizing and storing glycogen.

Likewise in the phlorhizinized animal fairly conclusive results have been achieved. Although the injection of this glucoside into normal animals does not appear to exhaust completely the glycogen reserves (44), the depletion is very great. A small residuum is held in the tissues so tenaciously, in fact, as to lead Ringer, Dubin, and Frankel (45) to differentiate it from the mobile portion. Cori found (36) that the administration of insulin to the phlorhizinized animal increased considerably the amount of hepatic glycogen. Page's work (46) supports that of Cori. These and many other similar investigations, which have been well reviewed by Nash, indicate that insulin aids the deposition of liver glycogen in the phlorhizinized animal.

Another useful technique has been the injection of glucose along with much insulin, thus magnifying the relatively small changes in glycogen distribution which insulin might be expected to produce in the normal animal. It is therefore of some interest that Cori and Cori (47) have reported that more glycogen was deposited after fructose administration than after glucose, but that large doses of insulin almost completely suppressed the formation of glycogen from either. This negative effect of insulin on the

deposition of hepatic glycogen in sugar-fed animals was observed during glucose absorption in the postabsorptive state, and in the adrenalectomized animal. Macleod had already shown in 1924 (48) that when insulin is given with glucose to "glycogen-free" rabbits, it retards glycogen synthesis and if given to "glycogen-rich" animals causes a decrease in glycogen content. In eviscerated cats, however, Best, Dale, Hoet, and Marks demonstrated that much of the increase in sugar utilization induced by insulin was due to the deposition of muscle glycogen (49). It was also shown (50) and confirmed by Choi (51) that unless the infusion of glucose was accompanied by insulin no increase in muscle glycogen occurred. An increased deposition of glycogen both in liver and muscle was reported by Collazo, Händel, and Rubino (52), and in the animal as a whole by Lesser (53), Brugsch and Horsters (54), and Bissinger, Lesser, and Zipf (55).

The hepatectomized animal has also proved of great value in insulin-glycogen studies. Removal of the liver was shown by Bollman, Magath, and Mann (56) to cause hypoglycemia and a marked decrease in muscle glycogen. Soskin (57) in confirming this work demonstrated also that muscle glycogen could hardly be considered as a source of blood sugar inasmuch as epinephrine failed to cause hyperglycemia in the hepatectomized animal. In recent work Markowitz, Mann, and Bollman (58) reported that the administration of glucose nevertheless led to a definite increase in muscle glycogen. If both the liver and pancreas were removed no rise in muscle glycogen was observed on the injection of glucose. The administration of insulin permitted the formation and deposition of muscle glycogen.

Finally the use of epinephrine in insulin studies has led to a number of important conclusions on the effect of insulin on glycogen metabolism. Macleod, Noble, and O'Brien, who were investigating the influence of insulin and epinephrine on the glycogen content of the liver during hypoglycemia, were the first to report that rabbits injected with these two hormones had more hepatic glycogen than if no insulin were given (59). It has been long suspected, of course, that epinephrine and the pancreatic hormone acted antagonistically,—the former inducing hyperglycemia and the latter hypoglycemia, and the usual glyco-

genolytic action of epinephrine being inhibited by insulin.⁵ In the course of the experiments of Part I it occurred to us that by the administration of insulin to standard epinephrine-treated animals in various physiological states further light could be thrown on the question of insulin-epinephrine antagonism and the action of insulin. The physiological states which we selected somewhat arbitrarily were four in number, the first being the condition of hyperglycemia and minimum glycogen concentration

TABLE V.
Effect of Insulin on Glycogen Content of Fasted Rabbits.

Rabbit No.	Weight.	Blood sugar.		Liver glycogen.	Muscle glycogen.	Remarks.
		Initial.	1 hr.			
	kg.	per cent	per cent	per cent	per cent	
62	2.5	0.100	0.061	0.52	0.35	2.5 units insulin per kilo after fasting 24 hrs. Killed 1 hr. after insulin.
63	2.5	0.100	0.052	0.26	0.49	
64	2.5	0.103	0.055	0.42	0.33	
11	2.0	0.100		0.47	0.62	Control animals. Fasted 24 hrs. No insulin administered.
12	2.0	0.103		0.43	0.50	
21	2.2	0.105		0.38	0.40	
22	2.2	0.100		0.50	0.47	

in the liver (1½ hours after epinephrine), the second, hyperglycemia and increasing liver glycogen (3 and 5 hours after epinephrine), the third, maximum liver glycogen and normal blood sugar (17 to 18 hours after epinephrine), and the fourth, decreasing liver glycogen and very low muscle glycogen (24 hours after epinephrine). Reference to Table III will make clear the reasons for selecting these particular times. It should also be noted that within a few hours after administering epinephrine the glycogen content of rabbit skeletal muscle falls to a very low but relatively constant level, thus permitting the observation of small ex-

⁵ It appears also that the peripheral vasoconstriction produced by epinephrine is opposed by a vasodilator effect of insulin. Certain it is that while one experiences great difficulty in bleeding rabbits from the marginal ear veins for about 3 hours after epinephrine, animals, which on the contrary, have received insulin bleed more freely than normal untreated controls.

perimentally induced changes which would otherwise escape detection.

Results.

Experiment 5.—In this experiment insulin was administered to three rabbits following a 24 hour fast. 2.5 units per kilo were injected intravenously and the animals killed 1 hour later. Lilly's insulin, U-100, diluted 1:50 was used. No epinephrine was given. The results, which are presented in Table V with control data from Part I, show quite clearly that the glycogen changes induced by insulin were too small to permit detection under these conditions. This agrees with the findings of McCormick and Macleod (33).

Experiment 6.—In Experiment 3 it was shown that 1 to 2 hours after injection of epinephrine the glycogen content of both liver and skeletal muscle was greatly reduced. Therefore, under these circumstances, the situation may be considered in some respects parallel to that of either the depancreatized animal or the true diabetic. The obvious similarities are (a) hyperglycemia, (b) glycosuria, and (c) low tissue glycogen. On the other hand there are several dissimilarities: (a) the pancreas in these epinephrine-treated animals is intact, and (b) vasoconstriction prevails in the peripheral vessels. As to whether or not epinephrine alters the rate of secretion of insulin is not well established, although the interesting work of Zunz and La Barre indicates an accelerating effect (60).

It has been shown by other investigators that epinephrine given simultaneously with insulin prevents hypoglycemia; further, that insulin given to epinephrinized animals prevents hyperglycemia and glycosuria. It seemed desirable therefore to determine whether or not insulin in this state of "epinephrine diabetes" causes a deposition of glycogen in the tissues.

Into each of three rabbits which had been fasted 24 hours, 1 mg. of epinephrine was injected. Half an hour later insulin (5 units per kilo) was administered intravenously. Blood samples for the estimation of sugar were drawn before the epinephrine was given and 1 hour after the insulin injection. At that time the animals were killed.

The insulin was given in non-convulsive doses, the object being

merely to introduce enough to prevent hyperglycemia. This was accomplished in two of the rabbits which were killed $1\frac{1}{2}$ hours

TABLE VI.

Effect of Insulin on Glycogen Content of Fasting Epinephrinized Rabbits.

Time of killing, after epinephrine.	Control animals. Epinephrine-injected, insulin not given.				Experimental animals. Both epinephrine and insulin given.				Insulin dosage.
	Blood sugar.		Liver glycogen.	Muscle glycogen.	Blood sugar.		Liver glycogen.	Muscle glycogen.	
	Before epinephrine.	At time of killing.			Before epinephrine.	At time of killing.			
hrs.	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	
1½	0.098	0.308	0.065	0.033	0.098	0.200	0.372	0.080	5 units per kilo ½ hr. after epinephrine.
1½	0.105	0.308	0.077	0.045	0.095	0.095	0.810	0.053	
1½	0.100	Lost.	0.079	0.030	0.091	0.105	0.155	0.103	
3	0.105	0.235	0.90	0.037	0.100	0.220	0.740	0.045	5 units per kilo 1½ hrs. after epinephrine.
3	0.091	0.220	1.03	0.062	0.105	0.240	0.795	0.038	
3	0.091	0.285	0.40	0.050					
3	0.100	0.335	0.44	0.036					
5	0.111	0.250	1.63	0.026			2.30	0.040	5 units per kilo 1½ hrs. after epinephrine. 5 units per kilo 4 hrs. after epinephrine.
5	0.110	0.180	2.29	0.030			2.25	0.037	
5	0.118	0.410	0.85	0.086			2.50	0.035	
18		0.098	3.89	0.030	0.095	0.033	1.40	0.038	3 units per kilo 17 hrs. after epinephrine.
18		0.105	3.73	0.031	0.098	0.037	1.65	0.039	
18			3.50	0.056	0.100	0.035	3.65	0.047	
25		0.100	0.265	0.023		0.038	1.24	0.245	2 units per kilo 24 hrs. after epinephrine. 5 units per kilo 24 hrs. after epinephrine.
25		0.100	0.900	0.024		0.058	3.00	0.016	
25		0.098	0.465	0.026		0.042	2.25	0.047	
						0.033	1.95	0.040	

after epinephrine administration, although a high blood sugar persisted in the third.

Under these conditions insulin caused a definite deposition of liver glycogen. Glycogen was also deposited in the muscles in small but significant quantities.

In the next group of experiments the time of action of epinephrine was steadily lengthened, permitting us to observe the action of insulin under the different physiological states referred to above. All of the results with control data from Table III are presented in Table VI.

Discussion of Experiments with Epinephrine and Insulin.

By reference to Table VI it appears that the administration of insulin to epinephrinized animals during hepatic glycogenolysis ($\frac{1}{2}$ hour and 24 hours after epinephrine) reduces the rate of decrease of liver glycogen. In spite of individual variations it is quite evident that the liver glycogen of the insulin-treated animals in the first and last groups is more abundant than in the corresponding controls. From data of members of the other groups we feel less prepared to draw conclusions unless it be to suggest that at the time of maximum concentration of glycogen in the liver (18 hours after epinephrine) insulin may tend to reduce the glycogen content of that organ. The former effect about which we feel more confident can be most easily explained by attributing to insulin the power of inhibiting hepatic glycogenolysis. Although of necessity we consider glycogen synthesis and hydrolysis to be proceeding simultaneously under all conditions, it is apparent that changes in the concentration of hepatic glycogen indicate only the dominance of one process over the other. It appears reasonable, however, to expect that if insulin influences primarily the hydrolytic reaction, its inhibitory effect could be most readily demonstrated experimentally when glycogenolysis is the dominant reaction. If, on the contrary, glycogen synthesis is the dominant process, conditions are less favorable for studying the effect of agents which influence only the hydrolytic reaction. Hence we incline to the view that insulin exercises a hepatic function, that of inhibiting glycogenolysis. With respect to peripheral effects, the experiments indicate that insulin promotes the deposition of muscle glycogen. Although the absolute increases observed in these experiments are small, they are quite definite among the animals of the first and last groups ($1\frac{1}{2}$ and 25 hours after epinephrine). The muscle glycogen values of the remaining animals were uninfluenced by insulin. In general the results can be readily understood by assuming that insulin

increases the peripheral synthesis of glycogen from blood sugar. In both of these hepatic and extrahepatic functions insulin thus antagonizes epinephrine which we assume to promote hepatic glycogenolysis and to inhibit the peripheral utilization of blood sugar.

It occurs to us that much of the disagreement recorded in the literature with respect to the action of insulin on glycogen distribution may be due to two principal variations in the experimental conditions, one of which favors the domination of the hepatic function, and the other, the peripheral function. In experiments conducted during dominance of the former, an inhibition in hepatic glycogenolysis will be observed. The amount of liver glycogen will increase. If the peripheral function is exercised more fully an increase in the utilization of blood sugar by muscle with a consequent reduction in liver glycogen will be observed.

SUMMARY.

1. An investigation has been made of the relationship existing between the time of action of epinephrine in fasted rabbits and the resulting changes in glycogen distribution.

2. During the first $1\frac{1}{2}$ hours a general depletion of glycogen was observed, characterized by hyperglycemia and a marked decrease in both liver and muscle glycogen.

3. By the 3rd hour the amount of hepatic glycogen had risen to the normal fasting value. It continued to increase to the 18th hour, by which time a pronounced deposition had taken place. Thereafter it fell rapidly to the fasting level.

4. The glycogen of muscle suffered a sharp decrease during the 1st hour of epinephrine action and remained at a low level throughout the 42 hours of observation.

5. The administration of insulin to epinephrinized animals inhibited hepatic glycogenolysis and resulted in an increase in the quantity of liver glycogen. This relative increase was observed only during those periods when the liver glycogen of the control epinephrinized animal was decreasing. At other times, the quantity of liver glycogen was either unaffected or exhibited a tendency to decrease.

6. In muscle, in the early and late periods of epinephrine action, insulin induced the deposition of small quantities of glycogen.

7. These results and those of other investigators may be explained by the theory that epinephrine promotes hepatic glycogenolysis and decreases the peripheral utilization of blood sugar. Insulin, on the contrary, is pictured as an inhibitor of hepatic glycogenolysis and a promoter of the utilization of blood sugar by muscle.

8. It is also suggested that the dominance of one function over the other (hepatic or peripheral) will be determined in part by the time of action of the hormone.

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THE COMPOSITION OF THE CELLS OF CERTAIN BACTERIA WITH SPECIAL REFERENCE TO THEIR CARBON AND THEIR NITROGEN CONTENT.*

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The chemical composition of bacteria has attracted considerable attention during the past few years. Interest in this field is based both on scientific grounds and on the hope that in the case of the pathogenic bacteria, a better understanding of the structure of the cell will enable man to combat more effectively the disease-producing power of such cells. The older literature on the chemical composition of bacteria has been reviewed by Kruse (1), Kendall (2), and, more recently, by Buchanan and Fulmer (3). The recent work on this subject has been concerned largely with pathogenic organisms; viz., the tubercle bacillus (4, 5) and the pneumococcus (6, 7). The present paper deals with the composition of three kinds of widely different non-pathogenic bacteria.

EXPERIMENTAL.

Preparation of Samples.—The organisms used were: (1) *Rhizobium meliloti* 100, a strain of the nitrogen-fixing bacteria of alfalfa, (2) *Clostridium acetobutylicum* A, a strain of the industrially important acetone-butyl alcohol bacteria, (3) *Lactobacillus leichmanni* A, a typical lactic acid organism. The cells were removed from the medium by centrifuging in a Sharples supercentrifuge. If any precipitates formed on sterilizing, the medium was centrifuged and again sterilized before being inoculated. The bacteria, grown on agar, were washed from the surface of the medium with water and filtered through cotton to remove any particles of agar before

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the suspension of cells was run through the centrifuge. By means of these precautions, the cells were obtained free from foreign organic material. As a further check on the purity of the material most of the samples were examined microscopically immediately after centrifuging to detect any debris; only clearly staining bacterial cells were observed.

The samples of *Rhizobium meliloti* were grown either on agar, (Samples 1 to 3) or in liquid medium (Sample 4). The agar medium consisted of: MgSO_4 0.2 gm., NaCl 0.2 gm., K_2HPO_4 0.4 gm., CaCO_3 0.4 gm., mannitol 10.0 gm., yeast-water 5 cc., agar 16 gm., and water 1 liter. The liquid medium contained the same salts as the agar medium but the K_2HPO_4 was increased to 2.0 gm., sucrose was substituted for mannitol, and yeast-water was replaced by peptone (0.5 gm.). The yield of wet cells from the liquid medium was 1.6 gm. per liter.

The cells of *Clostridium acetobutylicum*, Sample 5, were grown in Speakman's medium (8) at 38° and yielded 0.85 gm. of wet cells per liter of medium. The preparations of *Lactobacillus leichmanni*, Samples 6 and 7, were grown at 38° in 5 per cent yeast-water with 1 per cent glucose added and, in the case of Sample 7, the culture was neutralized with sterile alkali after 2 days incubation.

All the preparations were dried to constant weight at 100° and then ground to a fine powder before samples were taken for analysis.

Analytical Methods.—The carbon content of the dried cells was determined by the micro method of Lochte (9) on samples weighing from 20 to 30 mg. Before any analyses were attempted, highly purified benzoic acid was analyzed to determine whether our technique was sufficiently accurate. Eight typical analyses gave carbon percentages ranging from 67.4 to 68.8, and averaged 68.5 per cent. The calculated value is 68.8 per cent. The nitrogen content of the samples was determined by the Kjeldahl-Gunning-Arnold method. For the fat determinations, 1 to 2 gm. of the samples were extracted with anhydrous ether for 18 to 20 hours in a Soxhlet extractor with ground glass connections, and then for the same length of time with chloroform.

TABLE I.
Carbon Content of Certain Bacteria.

Sample No.	Kind of organism.	C	C (ash-free).
		per cent	per cent
1	<i>Rhizobium meliloti</i> 100.	51.5	52.9
		51.1	52.5
		51.4	52.8
	Average.	51.3	52.8
2	<i>Rhizobium meliloti</i> 100.	54.2	55.7
		52.4	53.9
		52.8	54.3
	Average.	53.1	54.6
3	<i>Rhizobium meliloti</i> 100.	52.1	53.6
		52.1	53.6
		52.5	54.0
		51.4	52.9
	Average.	52.0	53.5
4	<i>Rhizobium meliloti</i> 100.	53.4	54.3
		53.2	54.1
	Average.	53.3	54.2
5	<i>Clostridium acetobutylicum</i> .	44.3	46.5
		45.5	47.6
		45.9	48.1
	Average.	45.2	47.4
6	<i>Lactobacillus leichmanni</i> .	43.7	48.3
		44.9	49.6
		42.0	46.4
		44.0	48.6
		42.2	46.6
	Average.	43.4	47.9
7	<i>Lactobacillus leichmanni</i> .	40.5	45.2
		41.6	46.4
		41.5	46.3
		41.2	46.0
	Average.	41.2	46.0

DISCUSSION.

The carbon content of the different samples is given in Table I. The figures show a considerable difference in the percentage of carbon between the first four and the last three samples. The analytical results indicate how greatly the composition of different types of organisms may differ. Kruse (1) who has tabulated the carbon content of many distinctly unrelated organisms, gives figures ranging from 48.9 to 53.8 per cent. Our results slightly exceed even these limits.

TABLE II.
Nitrogen Content of Certain Bacteria.

Sam- ple No.	Kind of organism.	Age of cul- ture.	N	N (ash- free).*	C (ash- free).*	Approx- imate N content of media.
		days	per cent	per cent	per cent	mg. per l.
1	<i>Rhizobium meliloti</i> 100.	15	4.30 4.21	4.38	52.8	20
2	" " 100.	23	4.48 4.44	4.59	54.6	20
3	" " 100.	31	4.73 4.80	4.91	53.5	20
4	" " 100.	21	4.80 4.77	4.87	54.2	90
5	<i>Clostridium acetobutylicum</i> .	3	10.72 10.73	11.24	47.4	900
6	<i>Lactobacillus leichmanni</i> .	2	10.2 10.2	11.27	47.9	500
7	" "	3	10.93 11.14 11.14 10.83	12.28	46.0	500

* Average values.

In Table II are given the results of the nitrogen analyses. The differences in nitrogen content are even more striking than those observed for carbon. The nodule bacteria contain only a third as much nitrogen as do the butyl alcohol and lactic acid bacteria. Since the nodule bacteria were grown on a nitrogen-poor medium and the other two species on a nitrogen-rich medium, it may appear at first sight that there is a direct relation between the nitrogen

content of the medium and the nitrogen content of the cells. That such a relation exists is made doubtful by the nitrogen figures for Sample 4. In this case the medium contained almost 5 times as much nitrogen as the medium in which Samples 1 to 3 were grown but the percentage of nitrogen in Sample 4 is no higher than

TABLE III.
Ether and Chloroform-Soluble Extract of Certain Bacteria.

Sample No.	Kind of organism.	Ether-soluble fat in dry bacteria.	Chloroform-soluble fat in dry bacteria.	Total fat.
		per cent	per cent	per cent
1	<i>Rhizobium meliloti</i> 100.	1.2	10.2	11.4
2	" " 100.	0.6	14.6	15.2
3	" " 100.	0.6	14.9	15.5
4	" " 100.	0.9	21.7	22.6
5	<i>Clostridium acetobutylicum</i> .	2.0	1.0	3.0
7	<i>Lactobacillus leichmanni</i> .	1.3	0.5	1.8

TABLE IV.
Composition of Bacterial Cells.

Sample No.	Kind of organism.	Moisture.	Ash.	Protein.	Fat.	Carbohydrate.		Total.
						By difference.	Calculated from C.	
		per cent	per cent	per cent	per cent	per cent	per cent	per cent
1	<i>Rhizobium meliloti</i> 100.	71.9	0.8	7.6	3.2	16.5	17.7	101.2
2	" " 100.	71.9	0.8	7.9	4.3	15.1	16.6	101.5
3	" " 100.	71.9	0.8	8.4	4.4	14.5	15.1	100.6
4	" " 100.	71.9	0.5	8.4	6.4	12.8	12.6	99.8
5	<i>Clostridium acetobutylicum</i> .	76.0	1.1	16.1	0.7	6.1	4.1	98.0
7	<i>Lactobacillus leichmanni</i> .	72.0	2.9	19.3	0.5	5.3	2.1	96.8

in the other three cases. As in the case of higher plants, it is probable that the nitrogen content of bacteria is determined primarily by the kind of species and not by the environment of the species. The nitrogen percentage of *Rhizobium meliloti* increases slightly with age, while the carbon percentage varies irregularly with this

factor. At 23 days of age, the carbon content was about 2 per cent higher than at 15 days, but at 31 days, the figure was 1 per cent lower than at 23 days.

Table III gives the percentage of fat extractable by ether and chloroform for six samples. The low amount of fat extractable by ether indicates that only a small portion of the total fat is in the form of simple lipids or true fats. The chloroform-extractable material is surprisingly high in the first four samples. Similar observations have been made concerning the fat content of *Azotobacter chroococcum* by Ranganathan and Norris (10) and an organism closely related to *Bacillus megatherium* by Lemoigne (11). The nature of the chloroform-soluble fat of *Rhizobium meliloti* is being further investigated. Samples 5 and 7 have a low chloroform-soluble fraction, and a low total fat content.

In Table IV the possible composition of the bacterial cells is given. Since the moisture was not determined in Samples 1 to 3, the percentage found for Sample 4 was used. The crude protein was calculated by multiplying the total nitrogen by the protein factor, 6.25. On the assumption that proteins contain 53 per cent carbon and fats 76.5 per cent (Sherman (12)), the carbon of the fats and proteins is subtracted from the total carbon, and the residue considered to be the carbohydrate carbon. When this remainder was divided by the average percentage of carbon in carbohydrate (polysaccharides), 45.0, the dividend was assumed to be the percentage of carbohydrate present in the cells. The carbohydrate percentage, obtained by difference as in the approximate analysis of plant materials, is also given. The results by the two methods of calculation differ by only 1 to 2 per cent. For the nodule bacteria the figures based on the carbon content are all higher than those obtained by difference, while with the other two organisms the converse is true. The total percentage is approximately 100 and indicates that the assumptions made in calculating the protein, fat, and carbohydrate content of the cells are essentially correct.

The outstanding fact revealed by Table IV is that the dry matter of the root nodule bacteria consists principally of carbohydrate or something that resembles carbohydrate in composition, while the butyl alcohol and lactic acid cells are composed mainly of protein. The moisture content of all three species is essentially the same.

SUMMARY.

The carbon and nitrogen content of three species of bacteria were determined. The following percentages based on dry matter were obtained: nodule bacteria, C 52.8 to 54.6, N 4.4 to 4.9; butyl alcohol bacteria, C 47.4, N 11.2; lactic acid bacteria, C 46.0 to 47.9, N 11.3 to 12.3.

The fat (lipid) content (dry basis) of the three organisms in order was 11.4 to 22.6, 3.0, and 1.8 per cent. Ether and chloroform were used as the extracting solvents. Most of the lipids were contained in the chloroform extract, hence it is believed that but little of the extracted material is true fat.

From the above data and the moisture determinations the approximate composition of the cells was calculated. The moisture content of the three organisms was essentially the same. The dry matter of the nodule bacteria consisted chiefly of carbohydrate, and that of the other two species mainly of protein.

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ENZYMATIC HYDROLYSIS OF GLYCOGEN.

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The products of the enzymatic hydrolysis of glycogen have been assumed in the past to be maltose and glucose arising from intermediate dextrans and maltose. Although the disappearance of glycogen under the action of pancreatic amylase has been studied carefully by Norris (1) and the effect of salivary amylase has been investigated by Rona and Van Eweyk (2), there has apparently been no published account of an attempt to isolate or identify the product. It has been usually assumed to be maltose. Meyerhof and his collaborators (3) studied the production of lactic acid in digests of glycogen with ground frog muscle or muscle extracts, and Lohmann (4) has described the formation of a sugar in these digests which he believes to be similar to, or identical with, the "amylotriose" which Pringsheim (5) obtained as a product of the breakdown of glycogen and amylopectin with concentrated hydrochloric acid.

The importance of glycogenolysis merits a more detailed study of the process. An attempt was therefore made to obtain further information concerning the phenomenon from a study of the enzymatic hydrolysis *in vitro*. The first step was the preparation of glycogenase under standard conditions. Some of the factors controlling the hydrolysis were then studied, including an exploration of the possibility of reversion, and finally the end-products with different enzymes were identified.

An extract of rabbit muscle with glycerol was employed for the hydrolysis, since this extract contains an enzyme which is a probable factor in the normal breakdown of glycogen in the animal organism. It was found that this glycerol extract did not deteriorate during 6 months in the refrigerator, and was not inactivated by 24 hours incubation at 37°.

Stability of the Enzyme.

The enzyme was prepared by the method of Hunter and Dauphinee (6), the muscle being ground in a meat chopper as finely as possible before extraction. To determine whether the enzyme undergoes autodestruction, the following experiment was made.

A mixture of equal volumes of muscle extract and 0.25 M phosphate buffer of pH 6.3 was prepared. A 10 cc. sample was taken immediately after mixing with buffer and incubated with 10 cc. of 2 per cent glycogen for 2½ hours. The enzyme mixture was incubated for 48 hours at 37°. At intervals 10 cc. samples were taken and incubated with 10 cc. of 2 per cent glycogen for 2½ hours. The glycogen was determined in these digests before and

TABLE I.
Stability of Glycogenase at 37° at pH 6.3.

Sample No.	Period of incubation of enzyme.	Hydrolysis in 2½ hrs.
	hrs.	per cent
1	0	43
2	2	46
3	4	45
4	6	42
5	8	48
6	29	45
7	48	34

after incubation, with the results shown in Table I. There is no autodestruction of the enzyme on incubation at 37° for periods up to 24 hours. A slight loss in potency is observed in 48 hours.

Preparation of Glycogen.

The glycogen used was prepared and purified by a modification of the method of Pflüger (7). The livers of healthy rabbits were found to be the best source of material. The animals were well fed with carrots, and about 4 hours later were killed by a blow on the head. The livers were removed as quickly as possible, and immediately covered with an equal weight of caustic potash solution of sp. gr. 1.44 (42 per cent by weight KOH). The mixture was heated for 3 hours on a boiling water bath, cooled, diluted with an equal volume of water, and the glycogen precipitated by

the addition of sufficient 95 per cent alcohol to make the final concentration of alcohol 66 per cent. After standing for a short time, the supernatant fluid was decanted from the sticky precipitate, and the latter was dissolved in a quantity of water equal to the volume of the original solution. This solution was heated to boiling and filtered through a cotton or asbestos plug. The glycogen was again precipitated with alcohol. This second precipitate, after the supernatant fluid was decanted, was redissolved in the same volume of water, and carefully adjusted with glacial acetic acid to the turning point of phenolphthalein. 2 drops of glacial acetic acid were added in excess, and the solution heated to boiling and filtered through paper to remove the brown flocculent precipitate which separates. The filtrate was again heated to boiling and precipitated with *boiling* alcohol. The supernatant liquid was decanted, the precipitate dissolved in cold water in which it is readily and completely soluble, and precipitated with cold alcohol. This last precipitate was washed by decantation with increasing concentrations of alcohol, collected on a Buchner funnel, washed with absolute alcohol, and dried in an open dish over boiling water.

The product obtained in this way is ash-free, and consists of 99 to 100 per cent glycogen monohydrate ($C_6H_{10}O_5 \cdot H_2O$)_n, which according to Slater (8) is the stable form of glycogen at ordinary temperatures.

Identity of Glycogen from Different Sources.

The question of the identity of glycogen from different sources has been discussed at length by Norris (1) who describes experiments showing differences in the rate of hydrolysis of glycogen obtained from dog liver, rabbit liver, scallops, and yeast. In order to ascertain whether glycogen from these different sources is hydrolyzed at different rates under optimum conditions, the following experiments were carried out with equal concentrations of glycogen from scallops, rabbit liver, and rabbit muscle.

Solutions were prepared containing 6 cc. of 1 per cent glycogen, 3 cc. of phosphate buffer, and 3 cc. of glycerol extract of liver. After incubation for 1 hour, samples were taken and the protein precipitated by the method of Folin and Wu (9). The sugar in the filtrates was determined. The results, expressed as trisaccharide, are shown in Table II.

These results show that the rate of hydrolysis is approximately the same for rabbit liver and rabbit muscle glycogen, and somewhat lower for scallop glycogen. The results also show that there is no significant variation in the rate of hydrolysis caused by changing the C_{H^+} during the 1st hour of incubation. Judged by the criterion of rate of hydrolysis liver and muscle glycogen from the same animal are identical, and scallop glycogen differs from mammalian glycogen. The product of hydrolysis of scallop glycogen with muscle glycogenase is, however, identical with that of liver glycogen.

TABLE II.

Hydrolysis at Varying Hydrogen Ion Concentrations with Liver Extract of Glycogen from Different Sources.

pH	6.0	6.2	6.4	6.6	6.8	7.0
Source of glycogen.	Trisaccharide formed.					
	gm.	gm.	gm.	gm.	gm.	gm.
Liver.....	0.146	0.155	0.155	0.165	0.165	0.161
Muscle.	0.155		0.200	0.181	0.181	0.178
Scallop..	0.116	0.132		0.113	0.113	0.100

Estimation of Glycogen.

The determination of glycogen was carried out by a modification of the method of Pflüger (10) and Imamura (11). A convenient quantity for the determination is 5 cc. of solution. The sample is treated with an equal weight of a solution of KOH of sp. gr. 1.44, and heated on a boiling water bath for 3 hours under an air condenser. The resulting mixture is treated with an equal volume of water, and a sufficient volume of 95 per cent alcohol is added to make the final concentration of alcohol 66 per cent. The sample is then allowed to stand overnight to complete the precipitation of the glycogen.

The supernatant liquid is decanted through a filter paper, care being taken that as little as possible of the precipitate gets on the paper. The precipitate is washed by decantation, once with 20 cc. and then twice with 10 cc. of a saturated solution of sodium chloride in 66 per cent alcohol. The precipitate is then dissolved in hot water, in the same flask. The hot water is poured in through the filter, in order to dissolve the last trace of glycogen.

The solution, which should be approximately 50 cc. is heated on the water bath, under an air condenser, with the addition of 3 cc. of concentrated hydrochloric acid, for 3 hours. It is then cooled, a drop of phenolphthalein added, neutralized with concentrated KOH, made slightly acid with HCl, and made up to 100 cc. in a volumetric flask. The glucose was determined in the filtrate by the modification of the Shaffer-Hartmann (12) method described below, further dilution being made if necessary. From the glucose content of this solution the glycogen content of the original solution can be calculated. To avoid confusion, the results of glycogen determinations are expressed in terms of glucose, *i.e.*, they are calculated as $C_6H_{12}O_6$.

TABLE III.

Comparison of Direct Hydrolysis and Pflüger Methods for the Estimation of Glycogen.

Solution No.	Glycogen by direct hydrolysis.	Glycogen by Pflüger method.	Difference.
	per cent	per cent	per cent
1	0.0197	0.0199	+0.002
2	0.0091	0.0090	-0.001
3	0.072	0.073	+0.001
4	0.052	0.052	0.000
5	0.033	0.030	-0.003
6	0.017	0.017	0.000

The accuracy and sensitivity of the method were determined as follows: Solutions of glycogen of varying concentrations were prepared. Samples were taken in triplicate and hydrolyzed at once by hydrochloric acid. At the same time the glycogen was determined in triplicate samples by Pflüger's method. The average results are given in Table III.

Modification of the Shaffer-Hartmann Method.

The original method of Shaffer and Hartmann was found to be unsatisfactory for very accurate work with small amounts of material. Solutions containing less than 0.002 per cent of glucose give no reduction with the reagent and only approximate results are obtained with 0.004 per cent. By the use of a modified reagent, employed by Professor Shaffer and suggested by him, in

which the potassium iodide is omitted, it is possible to estimate accurately as little as 0.0005 per cent of glucose. The procedure is identical with that of the original Shaffer-Hartmann method except that after the samples are cooled, 5 cc. of 1 per cent potassium iodide solution are added immediately before the addition of the sulfuric acid.

The method of heating in open test-tubes was discarded owing to the danger of reoxidation and also to difficulties in titration. The solutions were heated in 100 cc. flasks, closed with a stopper containing a Bunsen valve. Two types of valves were used; the first, a glass tube with a very small opening covered by a ring of cigarette drainage tube; the second, a thick walled rubber tube closed at the top, and with a $\frac{1}{2}$ inch slit in one side. Both types were equally satisfactory. A sugar-thiosulfate table was constructed, based on experiments in which known concentrations of pure glucose prepared by the method of Hudson and Dale (13) were used. The glucose content of solutions of glucose of unknown concentration, determined by this method, always gave results which were identical with those obtained by determinations of the optical rotation.

Determination of Optimum Hydrogen Ion Concentration for Hydrolysis.

Five mixtures were made, each containing 10 cc. of 5 per cent glycogen solution, 5 cc. of enzyme, and 5 cc. of phosphate buffer. Two controls were included containing 10 cc. of distilled water in place of the buffer and enzyme. Toluene was added to prevent bacterial decomposition. The mixtures were incubated at 37° for 5 hours, after which the glycogen content was estimated and the pH determined electrometrically. The results are shown in Table IV.

The optimum hydrogen ion concentration for hydrolysis is at a pH of approximately 6.3. This agrees closely with values obtained for other amylases by Rona and Van Eweyk (2), by Paechtner (14), and by Norris (1).

The velocity of the hydrolysis is fairly slow. With moderate concentrations of glycogen, and at the optimum C_{H^+} , complete hydrolysis is effected in about 15 hours.

*Correspondence between Increase in Reducing Power and
Disappearance of Glycogen.*

During the study of the velocity of hydrolysis by muscle glycogenase, an exact correspondence was observed between the rate of disappearance of glycogen and the rate of appearance of reducing power of the digest. It was found that at any time during the course of the hydrolysis, the reducing substance which had ap-

TABLE IV.

Effect of Hydrogen Ion Concentration on Enzymatic Hydrolysis of Glycogen.

Sample No.	pH	Glycogen after incubation.	Hydrolysis.
		per cent	per cent
1	5.53	0.402	46
2	6.04	0.342	54
3	6.24	0.314	58
4	6.54	0.320	57
5	7.27	0.428	42
6	Control.	0.744	

TABLE V.

*Correspondence between Increase in Reducing Power and Disappearance
of Glycogen.*

Time.	Glycogen in 100 cc.	Reducing power, glucose equivalents in 100 cc.	Increase in reducing power, glucose equivalents in 100 cc.	Increase $\times \frac{100}{30}$, trisaccharide equivalents in 100 cc.	Glycogen destroyed in 100 cc.
hrs.	gm.	gm.	gm.	gm.	gm.
0	2.06	0.014			
3	1.80	0.091	0.077	0.25	0.26
6	1.57	0.233	0.142	0.47	0.49
12	1.08	0.291	0.277	0.92	0.98
24	0.44	0.510	0.496	1.64	1.62
36	0.16	0.660	0.646	2.13	1.90

peared, calculated as glucose, corresponded to almost exactly 30 per cent of the quantity of glycogen which had been destroyed during the same period. If the initial products of hydrolysis are dextrins, such a correspondence is improbable; rather, one would expect a rapid conversion of the glycogen into dextrin, followed by hydrolysis of the dextrin to reducing sugar. As will be shown

later, such a sequence occurs during the hydrolysis of glycogen by salivary and pancreatic amylases.

Results obtained in the hydrolysis of a 2 per cent glycogen solution are shown in Table V.

Identification of the Product of Hydrolysis.

Preparation of the phenylosazone of the product of hydrolysis of glycogen by the muscle enzyme gave a product consisting *entirely* of small star-shaped aggregates of needles. When purified, the osazone melts at 186° (corrected). This does not correspond to the melting point of the osazone of any sugar hitherto described as a product of the breakdown of glycogen. The same product was obtained whether the glycogen used was prepared from rabbit liver or from scallops. In view of opinions in the literature to the effect that since the melting points of the osazones extend over a range of several degrees and are not far apart for the different sugars, the determination of the melting points is of doubtful value, it may be stated that no difficulty was experienced in this respect. This compound, as well as other osazones which were prepared for comparison with it, on being rapidly heated to within about 10° below the melting point, the temperature then being raised slowly, began to darken somewhat at $2-3^{\circ}$ below the melting point, and then melted sharply within a range of less than 1° .

Considerable difficulty was experienced in obtaining a sufficient amount of the osazone for quantitative work. The material is not much more soluble in hot water than in cold, and is not readily crystallized from non-aqueous solvents. By making a very concentrated solution of the material in boiling pyridine or alcohol, and adding boiling water until precipitation commenced, it was found possible, however, to separate the osazone in pure form.

The molecular weight and nitrogen content of the osazone correspond to the values for a trisaccharide osazone of the composition $C_{30}H_{42}O_{14}N_4$.

On the assumption that only one product was present, determinations on glycogen digests showed that the product must have an optical rotatory power represented approximately by $[\alpha]_{H_2} = 182^{\circ}$ corresponding to $[\alpha]_D = 154^{\circ}$, with a reducing power 30 per cent of that of glucose.

Isolation of the Product of Hydrolysis.

The isolation of the sugar in the pure form presented considerable difficulty since the digests contained a complex mixture of protein, glycerol, and sugar. The first attempts consisted in the coagulation of the proteins by heat, or their precipitation by alcohol, concentration of the filtrate to the least possible volume; and precipitation of the sugar by absolute alcohol. This material was purified by acetylation and regeneration from the acetyl compound and was finally precipitated with absolute alcohol. The purified product showed $[\alpha]_{\text{H}_2\text{O}} = 187^\circ$, reducing power 8.5 per cent of that of glucose, and a molecular weight of approximately 481.

The low molecular weight and reducing power suggested that the sugar had been converted into an anhydride of the constitution $\text{C}_{18}\text{H}_{30}\text{O}_{15}$ with a theoretical molecular weight of 486, and this suggestion was supported by an analysis of the acetyl compound, showing 47.5 per cent $(\text{CO}\cdot\text{CH}_3)$ (theoretical = 47.5 per cent).

It is interesting to note that the anhydrotrisaccharide closely resembles the "grenzdextrin" obtained by Pringsheim and Beiser (15) from the digestion of amylopectin with malt amylase. The latter compound had $[\alpha]_{\text{D}} = 160^\circ$, the molecular weight of an anhydrotrisaccharide, and a reducing power by the Munson-Walker (16) method corresponding to 4 per cent of that of glucose.

The conversion of the sugar into its anhydride apparently takes place during the concentration of the glycerol-containing digest filtrate. This is shown by the fact that during this process the reducing power rises more slowly, in proportion, than the volume diminishes; and hence a conversion of the sugar into a compound of lower reducing power must be taking place.

The sugar itself was more effectively isolated by precipitation as a barium compound on the addition of excess barium hydroxide from a solution in 70 per cent alcohol. This compound was dissolved in water, decomposed by a current of CO_2 , the last trace of barium removed with sulfuric acid, and the filtrate evaporated to dryness. The sugar was purified by a repetition of this procedure, and showed a molecular weight of 505, an elementary composition corresponding to the formula $\text{C}_{18}\text{H}_{32}\text{O}_{16}$, $[\alpha]_{\text{H}_2\text{O}} = 181^\circ$, and reducing power 31 per cent of that of glucose.

Thus, the sole product of the hydrolysis of glycogen by a glycerol extract of muscle is a trisaccharide, with the physical constants given above, forming a characteristic osazone, melting at 186°. The characteristics of this compound differentiate it from the trisaccharides described by Pringsheim (5) and by Ling and Nanji (17).

Digestion with Other Amylases.

To compare the effect of muscle glycogenase with those of other amylases, digests of glycogen with salivary and pancreatic amylases were made. The results were quite different from those obtained with the muscle enzyme. In the case of salivary

TABLE VI.
Hydrolysis of Glycogen by Salivary Amylase.

Time.	Glycogen in 100 cc.	Glycogen destroyed in 100 cc.	Reducing power (as glucose) in 100 cc.	Ratio glycogen destroyed reducing power
<i>min.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
0	0.960	0.000	0.000	
10	0.876	0.084	0.010	8 4
20	0.750	0.210	0.027	7.8
40	0.478	0.482	0.093	5.2
60	0.304	0.656	0.160	4 1
120	0.134	0.826	0.200	4 1

amylase the increase in reducing power did not coincide with the disappearance of glycogen, being proportionately less at first and increasing steadily. The products also were different. Investigation of the products of hydrolysis by the osazone method showed that the characteristic osazone of the trisaccharide described above was absent. The chief product was isomaltose. Maltose could not be detected in any case, and glucose was only occasionally present. Since the reducing power of the digests was not at any time nearly enough to account for all the glycogen destroyed, it would appear that dextrins are formed also.

Hydrolysis by Salivary Amylase.

100 cc. of 1 per cent glycogen were treated with 0.5 cc. of filtered saliva at pH = 6.3 for 2 hours at 37°. Samples were taken at intervals for the determination of glycogen and sugar. The results are shown in Table VI. It is evident that the ratio of sugar formed is not constant, but increases continually during the course of the hydrolysis.

Treatment of the hydrolyzed residue with phenylhydrazine resulted in the formation of an osazone having the characteristic crystalline form of the osazone of isomaltose. No other osazone could be detected. The osazone when reprecipitated and dried melted sharply at 157° (corrected).

TABLE VII.
Digestion of Glycogen by Pancreatic Amylase.

Time.	Glycogen in 100 cc.	Glycogen destroyed in 100 cc.	Reducing power as glucose in 100 cc.	Ratio glycogen destroyed reducing power
min.	gm.	gm.	gm.	
0	2.47	0.00	0.00	
10	0.77	1.70	0.72	2.4
20	0.47	2.00	0.78	2.6
40	0.30	2.17	0.85	2.6
60	0.28	2.19	0.88	2.4
135	0.23	2.24	1.00	2.2

Hydrolysis with Pancreatic Amylase.

50 cc. of 4 per cent glycogen solution were treated with 0.02 gm. of Merck's trypsin and incubated for 2 hours at 37°, pH = 6.3. Samples were taken at intervals for glycogen and sugar determinations. The results are shown in Table VII.

The ratio of glycogen destroyed to reducing power with pancreatic amylase is constant and it therefore appears that there is no serial degradation of the glycogen, just as in the case of muscle glycogenase. The ratio, however, is different, averaging 2.4 in the case of pancreatic amylase and 3.3 in the case of muscle glycogen.

The residue from the hydrolysis with pancreatic amylase was heated with phenylhydrazine and acetic acid and the resulting osazone was examined microscopically. The characteristic crystals of isomaltose were identified as the principal product. Some crystals resembling those of glucosazone were also found. Repeating the procedure, with larger amounts of material, enabled a fraction to be separated which showed a melting point of 206° . The bulk of the material, when purified, melted at 157° .

In neither of the experiments quoted above could the characteristic crystals of the trisaccharide osazone be detected.

Effect of Increasing Glycogen Concentration on Hydrolysis.

Since the hydrolysis of glycogen belongs to the type which yields a number of products from the destruction of 1 molecule, it might be expected, as pointed out by Moore (18) that reversion of the reaction, *i.e.* synthesis, could be easily attained in concentrated solutions of the product. A corollary of this expectation is that in concentrated solutions of glycogen, hydrolysis would proceed at a slower rate than in dilute solutions. This has already been shown to be true for the hydrolysis of proteins (19). For the hydrolysis of glycogen by muscle glycogenase the velocity of hydrolysis was found to fall off rather rapidly with increasing concentrations of glycogen, varying from 80 per cent hydrolysis in 24 hours in a 1.20 per cent solution of glycogen to 70 per cent in a 2.32 per cent solution. In these experiments the concentration of the enzyme was kept proportional to the concentration of the glycogen, and was such a concentration that under the given conditions further addition of enzyme caused no increase in the rate of hydrolysis. Similar considerations would lead to the expectation that hydrolysis is readily retarded by the addition of products.

Effect of Addition of Products on Hydrolysis.

In the hydrolysis of glycogen by muscle enzyme it was found that the addition of the products of hydrolysis exerted a decided inhibiting effect. The addition of 5 per cent of the products was sufficient to reduce the rate of hydrolysis over 50 per cent, and the addition of 10 per cent practically inhibited the hydrolysis alto-

gether. This effect was observed both with the open chain trisaccharide and with the anhydride.

Since the products of hydrolysis inhibit the process, it was thought that in solutions containing a sufficient concentration of the trisaccharide and enzyme, it might be possible to attain the enzymatic synthesis of glycogen, as has been done in the case of protein. Nevertheless, under these conditions synthesis did not occur. Although attempts were made with solutions which varied in concentration from 20 per cent to the highest concentration obtainable, and at hydrogen ion concentrations from pH 3 to 11, no increase was noted in the glycogen content of any of these

TABLE VIII.

Rate of Hydrolysis of Glycogen by Muscle Glycogenase in Presence of Added Substance.

Added substance.	Average glycogen content.			Hydrolysis.	
	0 hr.	4 hrs.	24 hrs.	4 hrs.	24 hrs.
	per cent	per cent	per cent	per cent	per cent
Trisaccharide.....	2.02	2.00	1.92	0	5
Sucrose.....	2.02	1.74	0.84	14	58
Glucose.....	2.06	1.76	0.72	15	65
Sodium chloride.....	2.09	1.64	0.34	21	84
Urea.....	2.09	1.64	0.48	21	77
Control.....	1.99	1.60	0.56	20	72

solutions. Even when solutions containing the sugar and enzyme were evaporated to a syrup *in vacuo* over P_2O_5 , and then held for a considerable time at 37° , no glycogen was found. The specific inhibition which is observed may not therefore be a simple concentration effect, but may be due, for instance, to a combination of the enzyme with the added products.

As controls for the experiments, where retardation of hydrolysis was observed on adding trisaccharide, experiments were performed in which the trisaccharide was replaced by other substances. These were weighed out in sufficient amount to make a 0.2 M solution. 3 cc. of enzyme were added, and the volume made up to 10 cc. The results are shown in Table VIII. It is evident from these results that the effect of trisaccharide is specific

and not merely due to osmotic effects, for solutions of equal molar concentrations of other substances caused relatively no inhibition. Sucrose and glucose showed a very slight inhibition, while a slight acceleration of hydrolysis was observed with sodium chloride and urea.

During the course of these experiments a curious effect was observed. It was found that the addition of moderately high concentrations of egg albumin to glycogen digests had the effect of markedly accelerating the rate of hydrolysis, both in the presence and absence of the trisaccharide. This effect is illustrated in the following experiment. 5 cc. samples of 2 per cent glycogen solution were taken and the following substances dissolved:

TABLE IX.
Effect of Addition of Protein on Rate of Hydrolysis of Glycogen.

Substance added.	Average glycogen content.			Hydrolysis.	
	0 hr.	4 hrs.	24 hrs.	4 hrs.	24 hrs.
	per cent	per cent	per cent	per cent	per cent
0.5 gm. trisaccharide.	2 20	1 96	1 40	11	36
0.5 " " + 0.5 gm. albumin.	2.28	1 86	1 16	18	49
0.5 " egg albumin.	2 10	1 09	0 08	48	96
Control.	2 12	1.70	0.34	20	84

(1) 0.5 gm. of trisaccharide, (2) 0.5 gm. of trisaccharide + 0.5 gm. of egg albumin, (3) 0.5 gm. of egg albumin, (4) control. 3 cc. of enzyme were added to each sample and sufficient water to make the volume 10 cc. They were incubated at 37° for 24 hours with the results shown in Table IX.

Chemical and Physical Properties of the Trisaccharide.

In view of the importance of the conclusion that a trisaccharide is the sole product of the hydrolysis of glycogen by muscle glycogenase, the chemical and physical properties of the trisaccharide were investigated as completely as possible and the following data were obtained.

Melting Point of the Osazone.—Three determinations gave: 186° (corrected), 186° (corrected), 187° (corrected); average 186°.

Nitrogen Content of the Osazone.—Analyses by the micro-Kjeldahl method (Cole's modification) gave: 8.8 per cent nitrogen, 8.6 per cent nitrogen; average 8.7 per cent.

Calculated for $C_{36}H_{42}O_{14}N_4$ (trisaccharide) = 8.22 per cent.

" " $C_{24}H_{32}O_9N_4$ (disaccharide) = 10.78 " "

Rotatory Power of Digest.—One digest gave: Concentration = 0.438 per cent (determined by acid hydrolysis and estimation of the glucose formed), 2 dm. tube. $\alpha = +1.56^\circ$; whence $[\alpha]_{D_g} = +178^\circ$.

A second digest gave: Concentration = 0.476 per cent, 2 dm. tube. $\alpha = +1.79^\circ$; whence $[\alpha]_{D_g} = +187^\circ$.

Anhydrotrisaccharide.

Molecular Weight.— $W = 10.0$ gm., $w = 0.0413$ gm., $\Delta = 0.032^\circ$, K (water) = 18.6; whence $M = 481$. Calculated for $C_{18}H_{20}O_{11} = 496$.

Rotatory Power.—Concentration = 0.4127 per cent, 2 dm. tube. $\alpha = 3.09^\circ$; whence $[\alpha]_{D_g} = 187^\circ$.

Reducing Power.—Concentration = 0.4127 per cent (diluted 1 : 40 for determination); reduction (as glucose) = 0.0017; reduction by the same concentration of glucose = 0.0206. The reducing power is 8.5 per cent of that of glucose.

Acetyl Derivative of the Anhydrotrisaccharide.

Molecular Weight.—(In benzene solution.) Sample 1. $W = 7.03$ gm., $w = 0.0758$ gm., $\Delta = 0.053^\circ$, K (benzene) = 49.0, $M = 996$. Sample 2. $W = 8.98$ gm., $w = 0.0696$ gm., $\Delta = 0.041^\circ$, $K = 49.0$, $M = 926$. Calculated for $C_{18}H_{20}O_{11}(CO \cdot CH_3)_{10} = 906$.

Acetyl Content.

1. 0.0255 gm. required 2.80 cc. 0.1 N NaOH. $(CO \cdot CH_3) = 47.5$ per cent.

2. 0.0566 gm. required 6.25 cc. 0.1 N NaOH. $(CO \cdot CH_3) = 47.6$ per cent.

Calculated for $C_{18}H_{20}O_{11}(CO \cdot CH_3)_{10} \cdot (CO \cdot CH_3) = 47.5$ per cent.

Trisaccharide (Open Chain).

Molecular Weight.—(In water.) $W = 11.53$ gm., $w = 0.1848$ gm., $\Delta = 0.059^\circ$, K (water) = 18.6, $M = 505$. Calculated for $C_{18}H_{32}O_{18} = 504$.

Rotatory Power.—Concentration = 0.052 per cent, 2 dm. tube. $\alpha = 1.82^\circ$, $[\alpha]_{D_g} = 181^\circ$.

Reducing Power.—Concentration = 0.502 per cent (diluted 1 : 40 for determination); reducing power (as glucose) = 0.0075; reduction by same concentration glucose = 0.0251. The reducing power is 31 per cent of that of glucose.

Ultimate Analysis.

1. 0.1215 gm. gave 0.1911 gm. CO_2 , 0.0697 gm. H_2O . C 42.96, H 6.37, O 50.67.

Glycogen Hydrolysis

2. 0.1020 gm. gave 0.1561 gm. CO_2 , 0.0580 gm. H_2O . C 42.74, H 6.31, O 50.95.

Calculated for $\text{C}_{18}\text{H}_{32}\text{O}_{18}$. C 42.86, H 6.35, O 50.79.

Barium Compound of the Trisaccharide.

1. 0.1570 gm. gave 0.0562 gm. BaSO_4 . Ba 21.0.

2. 0.1509 gm. gave 0.0521 gm. BaSO_4 . Ba 20.3.

Calculated for $\text{C}_{18}\text{H}_{32}\text{O}_{16} \cdot \text{BaO}$. Ba 20.8.

CONCLUSIONS.

1. The hydrolysis of glycogen may be readily carried out by means of a glycerol extract of fresh muscle or liver tissue. Such an extract does not diminish in potency on long standing in a refrigerator, and its activity is only slightly diminished after incubation for 48 hours at 37° .

2. The optimum hydrogen ion concentration for glycogen hydrolysis with the tissue enzyme is about pH 6.3.

3. The sole product of the hydrolysis of glycogen by muscle extract appears to be a trisaccharide. This compound possesses 30 per cent of the reducing power of glucose, by the Shaffer-Hartmann method, and is dextrorotatory, $[\alpha]_{\text{Hg}} = +181^\circ$. The sugar is rather readily converted into an anhydride having 8.5 per cent of the reducing power of glucose, and $[\alpha]_{\text{Hg}} = 187^\circ$.

4. The hydrolysis of glycogen by the muscle enzyme is greatly inhibited by the addition of the trisaccharide or its anhydride to the digest. Sucrose and glucose have a slight inhibiting effect, and sodium chloride, sodium or potassium phosphate, and urea have a slight accelerating effect. The addition of 5 per cent albumin produces a marked acceleration, both in the presence and absence of the trisaccharide.

5. Attempts at the enzymatic synthesis of glycogen from the trisaccharide and its anhydride were unsuccessful.

6. The digestion of glycogen by salivary and pancreatic amylases follows a different course. The trisaccharide is not formed by these enzymes. Glucose and isomaltose have been identified among the products of these hydrolyses, but maltose apparently is not formed.

The author wishes to express his indebtedness to Professor H. Wasteneys for suggesting this problem and directing the progress of the work, and to Dr. H. Borsook for many helpful suggestions.

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STUDIES ON THE INORGANIC COMPOSITION OF BLOOD.

III. THE INFLUENCE OF SERUM ON THE PERMEABILITY OF ERYTHROCYTES TO POTASSIUM AND SODIUM.*

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That the red blood corpuscles are impermeable to sodium and potassium under physiological conditions seems to be well established. Hamburger (4, 5) claimed that sodium and potassium passed across the corpuscle membrane when blood was exposed to changed tensions of CO_2 , and also when the concentration of the cations in serum was changed, either by the addition of salts of sodium or potassium, or by dilution with water. A number of investigators have demonstrated that changes in CO_2 tension within the limits found in the human body cause little if any transfer of base between corpuscles and serum. This evidence has been reviewed by Wakeman, Eisenman, and Peters (12). These workers also disputed the claim of Hamburger (4, 5) that a transfer of sodium and potassium took place between serum and corpuscles when the concentration of cations in the serum was altered within physiological limits.

A number of investigators, however, have presented evidence that the corpuscles become permeable to cations when exposed to conditions beyond the physiological range. Mond (7) reported that red blood cells become permeable to cations when the alkalinity of the medium exceeds pH 8.0 to 8.3. Ashby (1) found enormous changes in the mineral composition of red blood cells when they were exposed to isotonic solutions of KCl and NaCl.

It is important to determine what deviations from physiological

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conditions result in an altered permeability of the red blood cells to the cations, since many studies are conducted under circumstances that are not physiological. The experiments presented in this paper show that the permeability of the corpuscles depends to some extent upon the proportion of serum present when the cells are exposed to changed concentrations of sodium and potassium.

EXPERIMENTAL.

Experiments were performed on dog, sheep, beef, and human red blood corpuscles. In each case a study was made of the effect of suspending the corpuscles in solutions containing varying proportions of serum and varying amounts of added KCl or NaCl. The corpuscles, freed from serum, were then analyzed directly to determine what changes had taken place in their content of potassium, sodium, and water.

Blood was obtained by heart puncture from dogs, and from the slaughter-house in the case of sheep and beef blood. It was defibrinated by gentle stirring, filtered through gauze to remove shreds of fibrin, and immediately centrifuged at about 3500 R.P.M. for 40 to 50 minutes. Losses of CO_2 undoubtedly occurred during these procedures, although this was avoided as much as possible. The human blood was collected and defibrinated under oil, losses of CO_2 being avoided in a more effective way than in the case of the other samples. After centrifugation, the corpuscles were separated as completely as possible from serum by aspirating off the latter, wiping the sides of the tube, and discarding the top layer of corpuscles with its admixed serum and white blood cells. The corpuscles were then mixed and divided into either 10 or 15 cc. portions, to each of which was added 30 cc. of the special serum-salt mixture in which the corpuscles were to be suspended. After allowing the suspension to stand 1 or 2 hours for equilibrium to become established, the corpuscles were again separated by centrifuging, the supernatant liquid discarded, and the corpuscles analyzed. In each experiment a specimen of the normal unwashed corpuscles was reserved for analysis.

The water content of the corpuscles was determined by drying specimens on cones of fluted filter paper in weighing bottles for 24 hours at 110° .

Protein-free filtrates for sodium and potassium determinations were prepared by first laking the samples (weighed in volumetric flasks) with water, then adding trichloroacetic acid to a concentration of 8 per cent, and diluting to definite volume.

Potassium and sodium were determined in ashed specimens of the trichloroacetic acid filtrates, potassium by the method of Shohl and Bennett (11), and sodium by the Kramer-Gittleman method (6). A preliminary study of the methods for sodium and potassium, with solutions of known composition, gave assurance of accuracy. Determinations were made in duplicate or triplicate, often at different times, and repeated if not in good agreement.

The results of the experiments are presented in the tables, Tables I to V dealing with dog blood. In all cases the normal unwashed sample of corpuscles is represented by A. All specimens from the same blood sample have the same number. Thus in Table IV three experiments are presented, Experiments 5, 8, and 7, representing different blood specimens. Hence comparison should be made only of specimens grouped under the same number.

Correction for changes in concentration caused by shifts of water was made by multiplying the values found for potassium and sodium by the factor $\frac{\text{H}_2\text{O in Specimen X}}{\text{H}_2\text{O in Specimen A (normal)}}$. These corrected results are presented in the columns headed K and Na, corrected.

The changes in cell volume were determined by measuring the volume occupied by the corpuscles in the centrifuge tubes. Since this was done in the wide 50 cc. tubes, the figures are only approximate and cannot be used in making accurate corrections for volume changes.

Experiments with Dog Corpuscles.

That the proportion of serum in the wash medium has an influence on the permeability of the red blood cells is evident from the results presented in Table I. The large increases in the potassium concentration of the corpuscles in the first series might be interpreted as due either to the decreasing proportion of serum present, or to the increasing concentration of potassium in the wash medium. In the second and third series, however, where a constant high concentration of potassium is maintained and the

TABLE I.
Effect of Variations in Proportion of Serum in Wash Medium on Permeability of Dog Corpuscles When Suspended in (a) Isotonic Serum-KCl Mixtures, K Varying Up to 600 Mg. per 100 Cc., and (b) Hypertonic Serum-KCl Mixtures, K Constant at 600 Mg. per 100 Cc.

15 cc. portions of corpuscles were mixed with 30 cc. of wash medium for 1 hour.

Experiment No.	Wash medium.					Red blood cells.										
	Contains per 100 cc.			Tonicity.	Mg. per 100 cc.		Per cent H ₂ O.	Mg. per 100 gm.		Mg. per 100 gm., corrected.		mm per 1000 gm., corrected.		Change in cell volume.	per cent	
	Serum.	1.15	KCl crystals.		K	Na		K	Na	K	Na	K	Na			K + Na
		cc.														
		cc.														
2-A	100			Iso.	20.4	303.0	66.51	54.2	243.1	13.9	105.7	119.6				
2-B	75	25		"	165	227.3	67.35	102.1	227.3	26.4	100.1	126.5	-6			
2-C	50	50		"	310	151.5	68.36	152.3	207.8	40.1	92.9	133.0	-5			
2-D	25	75		"	455	75.8	69.93	185.1	191.0	49.7	87.3	137.0	0			
2-E	0	100		"	600	0	67.93	208.5	172.5	54.5	76.6	131.1	+4			
2-A	100				20	303.0	66.51	54.2	243.1	13.9	105.7	119.6				
2-F	100		1106	Hyper.	600	303.0	57.14	120.8	302.2	26.5	112.9	139.4	-29			
2-G	75	25	830	"	600	227.3	59.70	122.4	273.7	28.1	106.9	135.0	-26			
2-H	50	50	553.3	"	600	151.5	61.86	164.7	244.0	39.2	98.7	137.9	-17			
2-E	0	100		Iso.	600	0	67.93	208.5	172.5	54.5	76.6	131.1	+4			
1-A	100				28.7	309.8	65.60	81.9	225.5	20.9	98.0	118.9				
1-B	100		1106	Hyper.	608.7	309.8	57.17	83.1	299.6	18.5	113.5	132.0	-22			
1-C	50	50	553	"	604.4	154.9	61.58	143.7	235.7	34.5	96.2	130.7	-13			
1-D	25	75	276.5	"	602.2	77.5	66.27	172.2	202.9	44.5	89.1	133.6	0			
1-E*	0	100		Iso.	600	0	68.39	158.5	172.5	42.3	78.2	120.5	+14			

* Slight hemolysis.

TABLE II.
Effect of Variations in Proportion of Serum in White Medium on Per cent of Dog Corpuscles When Suspended in Isotonic Serum-KCl-NaCl Mixtures, (a) with K Varying Up to 800 Mg. per 100 Cc., (b) K Constant at 800 Mg. per 100 Cc., (c) K Constant at 00 Mg. per 100 Cc.

In Experiment 9, 10 cc. portions of corpuscles were mixed with 30 cc. of white medium for 2 hours; in Experiment 8, 15 cc of corpuscles with 30 cc. of wash medium.

Ex- periment No.	Con- d.	0.85 per cent NaCl	Mg.	Mg.	00.	gm. l.	mm per 1000 gm., corrected.				Change in cell volume.		
							K	Na	K + N				
9-A 100			18.9	322.0	67.80	58.2	237.0	58.2	237.0	4.9	103.0	117.9	
9-B 70	Iso	15.67	99.2	277.4	68.03	51.9	247.0	52.1	248.0	3.3	107.8	121.1	0
9-C 70	"	7.33	149.2	249.5	67.26	54.7	235.8	54.3	233.9	3.9	101.7	115.6	1
9-D 69	"		199.2	222.0	67.82	65.4	250.3	65.4	250.3	6.7	108.8	125.5	-0
9-E 50	"	18.33	199.5	222.3	68.43	68.2	249.5	68.8	251.7	7.6	109.4	127.0	-1
9-F 25	"	42.50	199.7	222.5	68.54	61.8	237.4	62.5	240.0	6.0	104.3	120.3	-3
9-G 0	"	66.67	200	222.8	69.43	68.1	235.2	69.7	240.8	7.8	104.7	122.5	0
9-H 100	Hype		198.9	322.0	64.07	68.0	287.4	64.3	271.6	6.4	118.0	134.4	
8-A 100			20.0	319.2	66.87	38.0	242.6	38.0	242.6	9.7	05.5	15.2	
8-C 75	Iso.	10.83	00	286.8	66.79	37.1	250.9	37.0	250.4	9.5	08.9	18.4	0
8-D 50	"	35.00	00	276.6	67.43	36.3	253.2	36.6	255.2	9.3	11.0	20.3	+7
8-E 25	"	59.17	00	277.5	67.77	37.4	240.8	37.9	244.2	9.7	06.2	15.9	+1
8-F† 0	"	83.33	00	278.5	68.38	42.8	245.4	43.8	251.0	1.2	09.1	20.3	+3
8-B 100	Hype		00	319.2	65.76	37.0	259.6	36.4	255.2	9.3	11.0	20.3	-6

TABLE III.

Effect of Variations in Proportion of Serum in Wash Medium on Permeability of Dog Corpuscles When Suspended in Isotonic Serum-KCl-NaCl Mixtures, (a) with K Constant at 200 Mg. per 100 Cc., (b) 150 Mg. per 100 Cc.

10 cc. portions of corpuscles were mixed with 30 cc. of wash medium for 2 hours.

Experiment- No.	Wash medium.					Red blood cells.									
	Contains per 100 cc.			Tonicity.	Mg. per 100 cc.		Per cent H ₂ O.	Mg per 100 gm.		Mg. per 100 gm., corrected.		mm per 1000 gm., corrected.		Change in cell vol- ume.	per cent
	Serum.	1.15 per cent KCl.	0.85 per cent NaCl.		K	Na		K	Na	K	Na	K	Na		
cc.	cc.	cc.													
10-A	100				18.1	310.8	67.35	31.2	239.3	31.2	239.3	8.0	104.0	112.0	0
10-B	70	14.33		15.67	98	270.0	68.38	37.6	242.2	38.2	245.8	9.8	106.9	116.7	0
10-C	70	22.67		7.33	148	242.1	67.78	42.3	246.9	42.6	248.6	10.8	108.1	118.9	0
10-D	69	31.00			198	214.5	67.23	46.4	237.5	46.4	237.3	11.9	103.2	115.1	0
10-E	50	31.67		18.33	198	216.7	67.61	45.2	242.2	45.4	243.2	11.6	105.7	117.3	-5
10-F	25	32.50		42.50	198	219.7	67.76	47.1	227.1	47.4	228.9	12.2	99.5	111.7	-3
10-G	0	33.33		66.67	198	222.8	70.37	93.7	216.2	97.9	225.9	25.0	98.2	123.2	+7
10-H	100	343.3 mg.*			198	310.8	64.13	52.8	293.3	50.3	279.2	12.9	121.4	134.3	-15
10-I	100				18	310.8	67.35	31.2	239.3	31.2	239.3	8.0	104.0	112.0	
10-J	75	22.50		2.50	148	241.5	67.29	43.4	238.5	43.4	238.3	11.1	103.6	114.7	
10-K	50	23.33		26.67	148	244.5	67.73	41.7	242.3	42.0	243.8	10.7	106.0	116.7	
10-L	25	24.17		50.83	148	247.6	67.98	41.8	247.6	42.2	249.8	10.8	108.6	119.4	
10-M	0	25.00		75.00	148	250.7	68.94	57.3	Lost.	58.7	?	15.0	?	?	

* Crystals added instead of the isotonic solution.

proportion of serum is allowed to vary, the penetration of potassium into the corpuscles is obviously determined by the proportion of serum present, the permeability to potassium being greater the lower the proportion of serum in the wash medium.

With lower concentrations of potassium, variation in the proportion of serum has little influence on the permeability until the serum is entirely absent, when the penetration of potassium into the corpuscle is greatly increased (Experiment 8-F, Table II; Experiments 10-G and 10-M, Table III).

The transfer of potassium into the corpuscles depends not only upon the proportion of serum present, but also upon the concentration of potassium in the medium in which the corpuscles are suspended. The permeability of dog corpuscles to potassium was tested at levels of 600, 200, 150, and 100 mg. of potassium per 100 cc., with varying proportions of serum (Experiment 2, Table I; Experiments 8 and 9, Table II; Experiment 10, Table III). Penetration of potassium into the cells occurred at a level of 200 mg. in the presence of 69 per cent serum (Experiments 9-C and 9-D) and at a level of 150 mg. in presence of 70 or 75 per cent serum (Experiments 10-C and 10-J). At a level of 100 mg., potassium penetrated the cells in the presence of 70 per cent serum in one case only (Experiment 10-B), but in Experiment 8 (Table II) failed to penetrate except when serum was entirely absent. With a concentration of 600 mg. of potassium per 100 cc. in the wash medium, the corpuscle potassium increased far more when serum was absent than when present (compare Experiment 7-G with Experiment 5-F, Table IV), and in one case (Experiment 1-B, Table I) no potassium passed into the cells in the presence of 100 per cent serum. In the entire absence of serum the potassium concentration of the corpuscles showed a definite increase only when the concentration of potassium in the wash medium reached 100 mg. per 100 cc. (Experiment 7, Table IV).

The length of time the cells are washed seems to be of some importance (Table V), the concentration of potassium reaching a maximum in 2 hours, then decreasing. This has not been sufficiently studied.

A critical examination of the tables leads one to question whether many of the apparent changes in potassium concentra-

TABLE IV.
Effect of Varying K Content, (a) with Se m Plus Added KC₁H₄ per tonic, b) Isotonic KCl-NaCl Mixtures, in Absence of Serum.
 15 portion of corpuscle were mixed with 30 cc. of wash medium, 1 hour for Experiments 5 and 7,
 2 hours Experiment 8.

Experiment No.	Experiments	er [C]	To	Mg. per 100 cc.		Per cent H ₂ O.	Mg. per 100 gm.	Change in cell volume,						
				K	N.				per cent					
5-A	00			23.272	3.65	75	40.6	230.0	40.6	0.10	4.00	10.4		
5-B	00	38 mg. crystal	Hyper	43.272	3.65	37	44.6	246.3	44.3	244.8	11.3	06.4	17.7	
5-C	00	114 3 "	"	83.272	3.64	99	46.0	247.2	45.4	244.2	11.6	06.2	17.8	
5-D	00	267 0 "	"	163.272	3.63	25	59.9	264.7	57.6	254.6	14.7	10.7	25.4	
5-E	00	572 0 "	"	323.272	3.60	80	85.1	268.3	78.7	248.2	20.1	08.0	28.1	
5-F	00	184 3 "	"	143.272	3.58	62	40.4	299.3	125.2	267.0	32.0	16.0	48.0	
8-A	00			20.319	2.66	87	38.0	242.6	38.0	242.6	9.7	05.6	15.3	
8-H	00	57 2 mg. crystal	Hyper	50.319	2.66	47	37.8	275.0	37.6	273.4	9.6	18.9	28.5	
8-I	00	104 9 "	"	75.319	2.66	14	35.8	285.1	35.4	281.7	9.1	22.5	31.6	
8-B	00	152 6 "	"	00.319	2.65	76	37.0	259.6	36.4	255.2	9.3	11.0	20.3	
8-G*	†	190 7 "	"	708.3 mg			43.5	242.7	44.6	249.0	11.4	08.3	19.7	
7-A	00			?	?	66.09	42.6	250.3	42.6	250.3	10.9	08.8	19.7	
7-B	0	3 33 30.	5 %	Iso.	20.323	3.67	26	39.8	253.1	40.5	257.7	10.4	12.0	22.4
7-C	0	8 33 "	5 "		50.306	5.67	24	35.3	251.8	35.9	256.1	9.2	11.4	20.6
7-D	0	16 67 "	5 "		00.278	7.67	17	52.3	253.2	53.2	257.5	13.6	12.0	26.6
7-E	0	33.33 "	5 "		222.9	67.27		58.5	245.2	59.6	249.6	15.2	08.5	23.7
7-F	0	66 67 "	5 "		111.5	68.35		98.8	27.7	102.2	235.4	26.1	02.4	28.5
7-G†	0	100 "	5 "		0	68.68	218.7	20.3	227.2	125.0	58.1	54.4	12.5	+26

TABLE V.
Effect of Washing Dog Corpuscles with Serum Plus Added KCl for Periods Varying from 1 to 4 Hours.
 15 cc. portions of corpuscles were mixed with 30 cc. of wash medium.

Experi- ment No.	Time of washing.	Wash medium.					Red blood cells.								
		Contains per 100 cc.		Tonicity.	Mg. per 100 cc.		Mg. per 100 gm.		Mg. per 100 gm., corrected.		mm per 1000 gm., corrected.				
		Serum.	KCl crystals.]		K	Na	K	Na	K	Na	K	Na			
													cc.	mg.	
4-A		100		20	287			64.1	239.0	64.1	239.0	16.4	103.9	120.3	
4-B	1	100	1106	Hyper.	600	287			105.5	316.6	89.5	268.5	22.9	116.7	139.6
4-C	2	100	1106	"	600	287			139.4	312.5	117.4	263.1	30.0	114.4	144.4
4-D	3	100	1106	"	600	287			119.0	307.4	100.4	259.4	25.7	112.8	138.5
4-E	4	100	1106	"	600	287			113.6	267.7	96.9	228.3	24.8	99.3	121.1

tion may not be due to an incomplete separation of serum, rather than to an actual shift of potassium from the serum to the corpuscles. In the first group of results in Table IV, for example, the increase in the concentration of potassium in the cells amounts roughly to 10 per cent of that found in the serum. Thus an admixture of 10 per cent serum with the corpuscle sediment would account for the changes in potassium concentration in Experiments 5-B, 5-C, and 5-D (Table IV). This explanation however would necessitate unchanged values for sodium in the corpuscles, since the serum sodium is constant. The sodium concentration in the corpuscles however increased to much the same degree as potassium, although there is no added sodium in the serum. It is evident then that both potassium and sodium are being concentrated in the cells, either by entering from the serum, or because of the loss of some other part of the corpuscle substance. In Experiments 5-D and 5-E there was a decrease in cell volume of about 12 per cent, and in Experiment 5-F 25 per cent, whereas the water loss in these two cases was approximately 7.5 and 11 per cent. Hence only about half of the decrease in cell volume is accounted for by the water shift, so that a considerable shift of other corpuscle material must have taken place, concentrating the remaining constituents. Such a concentration explains the increase in sodium concentration, which is about 8 per cent in Experiment 5-E and 16 per cent in Experiment 5-F, but the increase of potassium in Experiments 5-D, 5-E, and 5-F can be explained only by a shift of potassium from serum to corpuscles. It may be mentioned here that Scott (10) found a loss of protein substance from corpuscles when suspended in Ringer's solution.

Further evidence that the changes in cell potassium are not due to admixed serum is found in the experiments in Table I in which the potassium concentration in the wash medium remained fixed, while cell potassium increased. Ege (2) states that complete separation of serum from corpuscles is accomplished when the centrifuged corpuscle sediment has the color of laked blood. This dark color was obtained in all our centrifuged specimens, except with sheep blood. Sheep corpuscles refused to pack even with 50 minutes centrifuging at 3500 R.P.M. With all other blood specimens the separation of serum from corpuscles is believed to be nearly complete.

Losses of sodium from the cells are observed (Experiments 10-F and 10-G, Table III) when the proportion of serum is low or absent, with the sodium concentration still at a fairly high level in the wash medium. Again in Experiments 2 and 1 (Table I) the marked losses of sodium might have been the result either of the low proportion of serum or the low concentration of sodium in the wash medium. In Experiments 7-F and 7-G (Table IV) the absence of sodium from the wash medium, rather than the absence of serum, seems to have been the factor causing the decreased concentration of corpuscle sodium. Marked increases of sodium in the cells when no changes have been made in the serum sodium are found in a number of cases, particularly in hypertonic solutions, where as already stated the cell volume has shrunk to a much greater degree than could be accounted for by loss of only water (*e.g.* Tables IV and V).

Experiments with Sheep, Beef, and Human Corpuscles.

The mixed corpuscles of two sheep were exposed to concentrations of 100, 150, and 200 mg. of potassium per 100 cc. of wash medium (Table VI). Increases in potassium and decreases in sodium concentration indicate that the sheep cells are somewhat permeable to both these cations, but the permeability seems to be little influenced either by the proportion of serum or the concentration of potassium in the wash medium, the penetration by potassium being the same at the 150 and 200 mg. levels. In Experiments 12-G and 12-L the high values for potassium in the cells must be discounted owing to the shrinkage of the cells in the hypertonic medium.

The most striking result in the experiments with sheep corpuscles is the apparently complete permeability of the cells when exposed to a concentration of 200 mg. of potassium per 100 cc. (Experiment 12-P, Table VI), in the entire absence of serum, the concentration of both sodium and potassium in the corpuscles becoming equal to that in the surrounding medium. The osmolar concentration increased about 40 per cent. There was no hemolysis.

The failure of potassium to penetrate to a greater extent in this series may be due to the fact that the concentration of potassium in the normal cells was already nearly equal to that in the wash medium.

TABLE VI
Effect of Variations in Proportion of Serum in Wash Medium on Permeability of Sheep Corpuscles When Suspended in Isotonic Solution
Serum-KCl-NaCl Mixtures, with K Constant at 100, 50, and 33 Mg. per 100 Cc
 10 cc. portions of corpuscles were mixed with 30 cc. of wash medium for 2 hours

E. No.	cc.	Tonic	Mg.	Per cent H ₂ O.	M ₁			M ₂			R. b.			K + Na	Color
					K	Ni	K	Ni	K	Ni	100 gm. fixed.	mm	h		
2-A	100		23.5	0	66.0		24.0		76.3	124.0	76.3	31.7	76.7	08.4	
2-B	100	Hypertonic	63.6 mg.	330.0	63.0		41.0		89.1	134.7	80.6	34.4	78.5	12.9	-9
2-C	69	Iso.	14.37 cc.	283.3	64.5		37.5		62.9	134.3	59.2	34.3	69.2	03.5	-4
2-D	50	"	15.00 "	282.0	65.0		36.8		54.4	134.7	52.1	34.4	66.1	00.5	-10
2-E	25	"	15.83 "	280.2	65.7		42.0		60.1	141.4	59.5	36.1	69.4	05.5	-1
2-F	0	"	16.67 "	278.4	66.6		36.2		58.8	137.4	60.2	35.1	69.7	04.8	-5
2-G	100	Hypertonic	247.9 mg.	330.0	62		61.0		89.6	152.8	79.9	39.1	78.2	17.3	-7
2-H	69	Iso.	22.70 cc.	255.4	64		34.7		55.6	131.3	51.7	33.6	65.9	99.5	-15
2-I	50	"	23.33 "	254.1	64		46.2		56.6	143.9	54.1	36.8	67.0	03.8	-9
2-J	25	"	24.17 "	252.4	65		44.3		58.5	143.0	57.1	36.6	68.3	04.9	-3
2-K	0	"	25.00 "	250.7	66		41.2		51.9	142.5	53.3	36.4	66.6	03.0	-5
2-L	100	Hypertonic	143.3	330.0	61		60.5		97.0	149.9	84.0	38.3	80.0	18.3	-16
2-M	69	Iso.	31.00	27.0	64		46.2		57.5	142.8	53.9	36.5	66.8	03.3	-6
2-N	50	"	31.67	26.3	64		44.1		55.3	141.7	52.7	36.2	66.4	02.6	-8
12-O	25	"	32.50 "	224.5	65		39.6		48.0	137.9	46.2	35.2	63.6	98.8	-6
12-P	0	"	33.33 "	200.0	65		42.4		22.6	201.8	221.9	51.6	96.5	148.1	-1

No hemolysis in any specimen.

* Crystals added instead of the isotonic solution.

TABLE VII.
Effect of Variations in Proportion of Serum in Wash Medium on Permeability of Beef Corpuscles When Suspended in
(a) Isotonic Serum-NaCl Mixtures, and (b) Isotonic Serum-KCl Mixtures.
 15 cc. portions of corpuscles were mixed with 30 cc. of wash medium for 2 hours.

Experi- ment No.	Wash medium.				Red blood cells.										
	Contains per 100 cc.			Tonicity.	Mg. per 100 cc.		Per cent H ₂ O.	Mg. per 100 gm.		Mg. per 100 gm., corrected.		mm per 1000 gm., corrected.		Change in cell volume.	per cent
	Serum.	1.15 per cent KCl.	0.85 per cent NaCl.		K	Na		K	Na	K	Na	K + Na			
		cc.		cc.											
13-A	100				21.7	306.4	66.49	63.4	185.5	63.4	185.5	16.2	80.7	96.9	
13-B	69		Iso.	31 00	15.0	315 0	65.52	60.5	187.8	59.6	185.0	15.2	80.4	95.6	-9
13-C	50		"	50 00	10 9	320 3	66.00	60 6	182.4	60.1	180.9	15.4	78.7	94.1	-9
13-D	25		"	75 00	5.4	327.3	66.30	57.6	181.7	57.4	181.2	14.7	78.8	93.5	-7
13-E	0		"	100 00	0	334.2	66.76	58.2	180.1	58.4	180.8	14.9	78.6	93.5	-4
13-A	100				21.7	306.4	66.49	63.4	185.5	63.4	185.5	16.2	80.7	96.9	
13-F	100	343.3 mg.*	Hyper.		201.7	306.4	61.79	85.5	200.8	79.4	186.5	20.3	81.1	101.4	-13
13-G	69	31 03 cc.	Iso.		201.2	241.4	65.60	80.9	176.0	79.8	173.5	20.4	75.4	95.8	-9
13-H	50	31.67 "	"	18.33	200.9	214.5	65.47	82.4	177.3	81.1	175.5	20.7	76.3	97.0	-5
13-I	25	32.50 "	"	42.50	200.4	218.6	66.15	81.7	179.1	81.3	178.2	20.8	77.5	98.3	-7
13-J	0	33.33 "	"	66.67	200.0	222.8	66.64	82.5	166.0	82.7	166.3	21.4	72.3	93.7	-5

* Crystals added instead of the isotonic solution

TABLE VIII.
Effect of Washing Human Corpuscles in Various Serum-Salt Mixtures.

10 cc. portions of corpuscles were mixed with 30 cc. of wash medium for 2 hours.

Experi- ment No.	Wash-medium.				Red blood cells.					
	Contains per 100 cc.			Tonicity.	Mg. per 100 cc. K	Per cent H ₂ O.	Mg. per 100 gm. K	Mg. per 100 gm., corrected. K	mm per per 1000 gm., corrected. K	Change in cell volume.
	Serum.*	KCl	0.85 per cent NaCl.							
				cc.	cc.	per cent				
14-A	100				17.4	65.94	333.9	333.9	85.4	
14-B	75			Iso.	13.1	66.05	336.5	337.2	86.2	-10
14-C	50			"	8.7	66.39	339.7	342.1	87.5	-5
14-D	25			"	4.4	65.04	324.6	320.1	81.9	-10
14-E*	0			"	0	62.46	288.8	273.5	70.0	-13
14-F†	0	100 cc. 1 15%.		"	100	69.96	387.9	411.5	105.2	+13
14-G	100	343.3 mg. crystals.		Hyper.	197.4	61.77	395.8	370.9	94.9	-14
14-H	{ 66.67 cc. serum. 33 33 " H ₂ O. }			Hypo.	11.6	72.05	279.0	304.9	78.0	+14

* The wash medium in Experiment 14-E, originally K-free, was found to contain 26.7 mg. of K per 100 cc. after being separated from the corpuscles.

† Slight hemolysis.

Washing beef corpuscles with serum-NaCl mixtures caused small losses of potassium from the cells (Table VII), while washing with serum-KCl mixtures (potassium at a concentration of 200 mg. per 100 cc.) caused a very definite increase of potassium in the corpuscles, apparently unrelated to the proportion of serum present.

Human red blood cells when washed with isotonic serum-NaCl mixtures lost potassium when the proportion of serum fell to 25 per cent or when it was entirely absent (Experiments 14-D and 14-E, Table VIII). A large increase in the potassium concentration occurred on washing the cells with isotonic KCl solution (Experiment 14-F). On adding KCl crystals to serum up to a concentration of 200 mg. per 100 cc. (Experiment 14-G) the potassium content of the corpuscles was raised. A large decrease of potassium resulted when the human corpuscles were suspended in serum diluted with half its volume of water (Experiment 14-H). It should be noted that if the results in Experiments 14-G and 14-H had been corrected for the change in cell volume rather than for the water shift, the corrected results would not be far from the normal value. Until further experiments can be made to study this point, the results in Experiments 14-G and 14-H should not be interpreted as contradictions to the results of Wakeman, Eisenman, and Peters (12), who found no transfer of base from corpuscles when washed in serum diluted with water, or in serum with added KCl. The decreased potassium concentration in Experiments 14-D and 14-E must however be due to a passage of potassium out of the corpuscles.

The figures for potassium in these experiments on human blood are in each case the averages of three closely agreeing determinations made at different times. Determinations on solutions of known potassium content were made simultaneously, so we are confident that these results are correct.

In each of the tables the sum of the concentrations of potassium and sodium has been recorded in terms of millimols per 1000 gm. Large increases above the normal value are noted in nearly every experiment, associated either with the entrance of potassium into the cell, or with a decrease in cell volume and condensation of the corpuscle material.

DISCUSSION.

The results obtained with dog corpuscles indicate unmistakably that the corpuscles become permeable to potassium and sodium when the proportion of serum surrounding the cells is lessened to a considerable extent. The serum inhibits the passage of cations across the cell membrane, but to what constituent of the serum this is due we have no evidence. Among the possible factors concerned are the serum proteins, lipoids, and the bivalent cation calcium. It is also possible that the protective influence may be merely that of a buffer, preventing shifts of pH which must occur to a greater extent in the absence of serum. This protective influence of the serum is evident in the experiments with sheep blood, although to a less extent, a marked transfer of both potassium and sodium taking place in the absence of serum in Experiment 12-P (Table VI). With beef blood there is evidence of increased permeability when the proportion of serum is less than normal, but the permeability seems to be no greater in the complete absence of serum than when 70 per cent serum is present. The protective action of the serum is definitely seen in the experiments with human blood, the corpuscles losing potassium as the serum is replaced with saline solutions.

The difference in results obtained with the blood of various species draws attention to the fact, so often ignored, that the corpuscles of different species differ greatly in mineral composition. It is a question whether conclusions based on work with one species may be applied to the blood of other species. For example the work of Wakeman, Eisenman, and Peters (12) on human blood cannot be assumed to disprove the different results obtained by Hamburger (4, 5) who used horse, pig, and beef blood.

The results obtained have little bearing on the permeability of red blood cells under physiological conditions. In a few experiments, changes in the potassium or sodium concentration in cells were noted when the changes in the serum were within limits occasionally found during life, but since CO₂ losses were not avoided, the serum undoubtedly became abnormally alkaline, approaching the conditions under which Mond (7) found the cells permeable to cations.

The discrepancy between the change in corpuscle volume and

the water shift in many cases has already been pointed out, and the author is aware that the method of correcting the results to the normal water content does not adequately correct for the volume changes. Neuhausen and Breslin (8) observed peculiar changes in cell volume in isoosmotic solutions, the greatest increase in volume occurring in a solution of KCl. In our experiments isotonic KCl solutions in a number of cases caused increased cell volume (Experiments 7-F and 7-G, Table IV; Experiment 1-E, Table I; Experiment 14-F, Table VIII), and in each of these samples there was a large increase in the concentration of potassium in the cells. In two of these (Experiments 7-F and 7-G, Table IV), the corpuscle sediment became very thick and pasty, in spite of the increase in volume. No record was made of the character of the sediment in the other cases.

The marked alterations shown to occur in chemical composition when the cells are washed with either isotonic KCl or NaCl is evidence that these solutions are not "physiological." The results obtained in Experiment 14-E (Table VIII) may explain the finding of Rous and Turner (9) that washing cells in NaCl or in Locke's solution injures them, but if some plasma is present the injury is less. Gough (3) noted that if human cells are added to isotonic saline they become spheres, the addition of serum causing them to reassume their discoid form. These observations are further evidence of the protective action of serum on the red blood cells.

It is a common procedure in many investigations to wash blood corpuscles, even repeatedly, in saline solutions, and also to preserve cells in saline for many hours. These procedures are certainly open to criticism, and in some cases must vitiate the results.

Our experiments confirm the results of Ashby (1), who found great changes in the mineral composition of corpuscles which had been suspended in isotonic solutions of NaCl and KCl.

SUMMARY AND CONCLUSIONS.

1. The permeability of dog, sheep, beef, and human red blood corpuscles has been tested to potassium and sodium in the presence of varying proportions of serum and varying concentrations of potassium.

2. The results indicate that the red blood cells become progressively more permeable to cations as the proportion of serum present decreases.

3. The transfer of potassium across the cell membrane depends also upon the concentration of potassium in the surrounding medium. As the proportion of serum increases, the level of potassium must be raised in order to enter the cells.

4. Sheep and beef corpuscles are less permeable than those of the dog, and the influence of serum is less evident.

5. Human red blood cells lose potassium when washed in isotonic saline solutions containing little or no serum, and gain potassium in isotonic KCl solution.

6. Attention is called to the fact that 0.85 per cent NaCl solution is not "physiological," since it permits transfer of ions with the red blood cells.

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STUDIES ON LIGNIN METABOLISM.*

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Food materials of vegetable origin constitute a large proportion of the diet of man and of animals. In many of the human foods, but more especially in the various animal feedingstuffs, lignin is present in some cases to the extent of 20 to 25 per cent of the dry weight of the food material. It is therefore rather surprising that such a common food ingredient has not been studied and subjected to metabolism experiments to a greater extent than we find recorded in the literature. The difficulties encountered in the quantitative estimation of lignin may have withheld many investigators from studying the metabolism of this substance.

There is an abundance of evidence to support the claim that lignin, unlike cellulose, is cyclic in structure, the nature of the ring being either aromatic or hydroaromatic. In support of this, it is sufficient to mention the work of Cross and Bevan (1903), Hochfelder (1915), Melander (1919), Honig and Fuchs (1919, 1920), Erdmann (1921), Fischer, Schrader, and Friedrich (1921), Strupp (1924), and that of Strache-Lant (1924).

Several investigators, among them Meissner and Shepard (1866), Harten (1867), Weiske (1876), and more recently König (1926), have suggested that lignin probably furnishes the phenyl radical involved in the formation of hippuric acid, but they all failed to prove their hypothesis experimentally. The last mentioned investigator working with Dietrich in 1871, as well as Stutzer (1875), has claimed, however, that lignin is not digested in the animal body. Recently, Paloheimo (1925) stated that lignin reappears quantitatively and unchanged in the feces.

* Preliminary notes on this subject were published in *Proc. Am. Soc. Biol. Chem.* (1928) and in *Proc. Soc. Exp. Biol. and Med.* (1929).

Rogozinski and Starzewska (1926, 1928) found that the lignin in oat straw is not digested by sheep. The methoxyl group determinations made on the feces also indicated that the lignin is not digested by these animals. Rubner (1928) in a more recent paper reported a loss of lignin in human beings as well as in dogs. Our experiments with dogs and cows point to a degradation of lignin. Our conclusions are based on the fact that an increase in the hippuric acid eliminated was obtained after lignin was ingested, as well as on the decrease of the methoxyl group content of the lignin eliminated.

The methods most commonly employed for the quantitative estimation of lignin are based on the removal, by hydrolysis with strong mineral acids, of the carbohydrates and other substances, leaving the lignin as an insoluble residue. In our opinion these methods are not sufficiently accurate to determine the fate of lignin in metabolism experiments. On the other hand, the methoxyl group which is known definitely to be present in lignin may be determined with a great deal of accuracy by the Zeisel (1923) method or by the various modifications of it, and it was thus possible to follow up any loss of the methoxyl group in our metabolism experiments with lignin. Experiments conducted with a dog showed a loss of 15 per cent of the methoxyl group initially present in the lignin and in an experiment with a cow a 37 per cent loss of the methoxyl group was observed. We believe that in these experiments we have direct evidence that lignin is metabolized in the animal body. Our results on the benzoic acid eliminated in the urine when lignin was fed support this claim.

Experiments were conducted *in vitro* in which known quantities of lignin mixed with fresh material taken from the four compartments of a cow's stomach were incubated. A loss of the methoxyl group was observed, indicating that the partial disappearance of the methoxyl group of the lignin observed *in vivo* takes place in the stomach of the animal and that it is not brought about by bacteria, but rather by some other agent, possibly by an enzyme in the gastric juice of the animal.

EXPERIMENTAL.

The lignin used in our experiments was isolated from corn cobs by the alkali method. The corn cobs were treated with 6 times

their weight of 2 per cent sodium hydroxide solution and heated in an autoclave for 8 hours under 25 pounds pressure. The alkaline liquors were then drained off, the cobs were washed with water, and the wash water was added to the alkaline liquor. This liquor was filtered and acidulated with an excess of hydrochloric acid to make an approximate 2 per cent solution of this acid. This was heated to boiling and boiled for 1 hour, allowed to cool to room temperature, then centrifuged in a basket centrifuge, and finally dried at 100°. The lignin thus obtained was amorphous, light brown in color, and free from cellulose.

All the methoxyl group determinations recorded in this paper were made according to the Kirpal and Bühn (1914, 1915) modification of the Zeisel method. To facilitate the splitting off of methyl iodide by the hydriodic acid, phenol was added to the reaction mixture as recommended by Weishut (1912). The iodine of the methyl iodide was determined gravimetrically as silver iodide.

We used both herbivorous (cow) and carnivorous (dog) animals in our experiments. We used dogs in our experiments because it is possible to keep these animals on an entirely lignin-free diet (meat), thus making it easier to show definitely the connection between the lignin intake and the benzoic acid elimination. Furthermore, meat and the feces of a dog kept on a meat diet are free from the methoxyl group, and any loss of the methoxyl group when lignin is ingested would necessarily show a chemical change of lignin. A cow would stop ruminating if roughage were excluded, and even so it is hardly conceivable to select lignin-free food for a cow. On the other hand, the urine of herbivorous animals contains normally rather large quantities of hippuric acid, and it was thought that the metabolism and utilization of lignin might be evidence of a specific adaptation of their digestive system.

The dogs used in our experiments were first kept for from 10 to 14 days on a meat and bone diet. At the end of that period a known amount of lignin mixed with 200 to 300 cc. of water was introduced with a stomach tube at 10.00 a.m. of each lignin feeding day, after which the animal was fed the meat and bone ration. The total benzoic acid content of the urine in all of our experiments was determined by the method of Kingsbury and

TABLE I.

Lignin Feeding Experiments with Dogs, Showing Effect on Hippuric Acid (Benzoic Acid) Elimination.

Experiment No.	Date.	Dog No.	Weight of animal.	Meat fed daily.	Lignin added to meat diet.	Urine analysis.	
						N	Total benzoic acid.
	1928		kg.	gm.	gm.	gm.	gm.
1	Feb. 14	1	12.5	150		10.97	0.357
	" 15			150	25	12.16	0.648
	" 16			150	25	9.42	0.879
	" 17			150	25	8.15	0.676
	" 18			150	25	10.37	0.795
	" 19			150	25	11.13	0.810
	" 20			150		8.35	0.359
	" 21			150		9.69	0.339
	" 22			Fasting.		2.49	0.207
	" 23				25	3.67	0.705
2	July 16	2	7.8	150		9.438	0.205
	" 17			150		7.250	0.209
	" 18			150	16	7.870	0.285
	" 19			150	12	8.370	0.272
	" 20			150	12	6.243	0.255
	" 21			150		6.648	0.116
	" 22			150		5.382	0.148
	" 23			150	16	5.024	0.274
	" 24			150	16	3.651	0.179
	" 25			150		6.263	0.189
	" 26			150		7.183	0.186
3	Dec. 12	3	7.25	200		8.473	0.145
	" 13			200	12	7.855	0.223
	" 14			200	12	8.259	0.205
	" 15			200	12	8.910	0.351
	" 16			200	15	10.10	0.295
	" 17			200	15	8.354	0.270
	" 18			200	15	8.559	0.265
	" 19			200	15	9.622	0.237
	" 20			200		7.670	0.192

Swanson (1921), and the total nitrogen by the Kjeldahl method. The dog feces collected in the fore period of the experiment were found to contain no methoxyl groups. The feces collected during

the lignin feeding period and the 2 day after period were combined, dried, and ground, and the methoxyl group content was determined. To convince ourselves that a 2 day after period was sufficient, we collected the feces for another day and determined their methoxyl group content. They were found, however, to be free of the methoxyl group, showing that all the lignin had been eliminated in the 2 day after period. The results of our experiments with dogs are recorded in Table I. We have omitted from

TABLE II.

Lignin Feeding Experiments with Cows, Showing Effect on Hippuric Acid (Benzoic Acid) Elimination.

Experiment No.	Date.	Cow.		Lignin added to basal diet.	Urine analysis.			
		Age.	Weight.		N	Average per day.	Benzoic acid.	Average per day.
		Yrs.	kg.	gm.	gm.		gm.	
4	1927							
	Nov. 8	2	430		49.70	54.67	30.17	32.34
	" 9				59.64		34.52	
	" 10			500	56.34	54.12	44.10	45.08
	" 11			500	51.91		46.06	
5	1928							
	Nov. 13	11	575		82.92	95.91	62.124	62.124
	" 14				108.9		62.124	
	" 15			500	97.70	93.76	65.823	64.851
	" 16			500	88.95		64.740	
	" 17			500	94.65	103.56	63.99	61.194
	" 18				83.72		57.168	
	" 19				123.4		65.221	After period.

Table I, however, the results of one experiment in which no increase in the benzoic acid elimination was observed. The dog in this experiment received 2.5 gm. of lignin per kilo of body weight, and this rather large amount of lignin produced decided toxic symptoms, so that the experiment had to be discontinued.

The results of our experiments with cows are given in Table II. The daily ration of the heifer in Experiment 4 consisted of 4 pounds of grain mixture and 10 pounds of beet pulp. The 2 year old heifer was kept on this ration for 2 days previous to our

experiments. The composition of the daily ration of the cow used in Experiment 5 is given in Table III. This diet was fed to the cow for 2 weeks previous to our experiment. The cow was catheterized at the beginning and at the end of the experimental period, so that the average figures per day given in Table II for urinary nitrogen and benzoic acid are more nearly correct than those given in the daily analysis. In Experiments 4 and 5 the urine was collected daily (from 10.00 a.m. to 10.00 a.m.). During the fore period, experimental period, and after period of Experiment 5, the feces were collected daily, thoroughly mixed, and an aliquot (0.001 of the weight) of each was taken. The aliquot samples of each period were combined, and the moisture and methoxyl group content determined. The results obtained when

TABLE III.

Composition and Methoxyl Group Content of Daily Ration of Cow Used in Experiment 5.

Feed.	Methoxyl content on moisture-free basis.	Amount in daily ration.	
		Feed.	Methoxyl.
	<i>per cent</i>	<i>kg.</i>	<i>gm.</i>
Hay.....	2 46	2.72	60 4
Grain mixture..	0 80	2.27	16.8
Silage.....	2 05	9.1	172.3
Lignin... ..	14 05		

multiplied by 1000 gave the methoxyl group elimination for each period.

The results in Table II show clearly the effect of lignin on the benzoic acid elimination. The increase in the average daily benzoic acid output in the lignin feeding period was not so high in Experiment 5 as in Experiment 4. However, the effect is definite.

To prove that the lignin is responsible for the increase of benzoic acid elimination, we had to convince ourselves that lignin undergoes some chemical change. A complete recovery of lignin in the feces would mean that this substance affects the benzoic acid elimination only indirectly, while a loss of lignin may be interpreted as indicating that it is the source of the hippuric acid. We first attempted to determine quantitatively the lignin fed to the animal and the lignin eliminated in the feces, and for this purpose

we employed the Dore (1920) modification of the Willstätter and Zechmeister (1913) method. This method, which is based on the fact that fuming hydrochloric acid (sp. gr. 1.21 to 1.22 at 15°) will dissolve all organic substances present in a lignified material except the lignin, was found to be unreliable when applied to the analysis of feces. We found that certain nitrogenous substances present in the feces also did not dissolve in the fuming hydrochloric acid. Furthermore, the lignin preparation which was used in our experiments when subjected to the action of fuming hydrochloric acid suffered a loss in weight of approximately 15 per cent. We accordingly had to resort to an entirely different procedure. Of the various constituent groups believed to be present in the lignin molecule, OCH_3 , or the methoxyl group has been definitely established. Knowing the methoxyl group content of the lignin used in our experiments, we were able by determining the percentage of the methoxyl group in the feces to ascertain whether or not the lignin is attacked by the animal body. In our dog experiments it was necessary to obtain only the percentage of the methoxyl group in the feces collected during the lignin feeding period and the after period in order to determine whether the lignin suffered any loss of the methoxyl group, as neither the food (meat) nor the feces in the fore period contained any methoxyl group. In the dog experiment we can claim definitely that a loss of the methoxyl group means a loss of the lignin methoxyl group. In the cow experiment, however, it was necessary to determine the methoxyl group content of each food constituent and that of the feces eliminated in the fore period (Table III) in order to ascertain whether any loss of the methoxyl group occurred.

Table IV gives the results on the methoxyl group balance in three of our experiments. It will be observed that in the two dog experiments a 20.3 per cent and 13.3 per cent loss of the methoxyl group occurred. In the experiment with the cow a loss of 36.7 per cent of the lignin methoxyl group was observed, while 60.8 per cent of the total methoxyl group present in the food disappeared. More evidence is needed to prove convincingly whether this process in the animal organism is in the nature of a demethoxylation or whether the lignin molecule is broken down to a greater extent. We may follow this problem up in the future

TABLE IV.
Methoxyl Group Balance in Metabolism Experiments.

Animal.	Fore period. Daily methoxyl:			Experimental period.				
	Intake in food.	Output in feces.	Loss.	Methoxyl in lignin added.	Total methoxyl output in feces.	Increase over normal output due to lignin.	Loss of methoxyl in lignin added.	
	gm.	gm.	per cent	gm.	gm.	gm.	gm.	per cent
Dog of Experiment 2.	None.			13 0	10.4	10.4	2.6	20.0
" " " 3..	"			13 48	11 67	11.67	1 81	13.3
Cow " " 5.	249 5	77.8	68 8	210 8	522 2	133 3	77 5	36.7

TABLE V.
Digestion Experiments in Vitro Showing Loss of Methoxyl Group of Lignin Incubated with Stomach Content of Cow.

Experiment No.	Material from stomach pouch No.	Stomach content used (moist weight).	Total methoxyl in sample of stomach content + 1 gm. lignin.*		Loss of methoxyl.		Remarks.
			Before incubation.	After incubation for 8 days at 38°.			
		gm.	gm.	gm.	gm.	per cent	
6	1	10.1764	0.0396	0.0398	0	0	Control, no lignin nor toluene added.
7	1	10.5289	0.0409	0.0405	0.0004	0.9	Control, toluene added.
8	1	27.0484	0.2532	0.2268	0.0264	10.4	Toluene added.
9	1	26.5842	0.2513	0.2305	0.0208	8.2	
10	2	17.1946	0.2095	0.1993	0.0102	4.8	Toluene added.
11	2	26.7502	0.2487	0.2387	0.0100	4.0	
12	3	12.0796	0.2193	0.2179	0.0014	0.64	Toluene added.
13	3	14.4198	0.2348	0.2341	0.0007	0.3	
14	4	29.8150	0.2712	0.2607	0.0105	3.87	Toluene added.
15	4	24.4406	0.2473	0.2351	0.0122	4.93	

* 1 gm. contained 0.1391 gm. of the methoxyl group.

and also determine whether any relationship exists between the loss of the methoxyl group and the increase in hippuric acid eliminated.

In order to locate the place in the digestive tract of the animal where the degradation of the lignin takes place, we conducted several experiments *in vitro*. Known quantities of lignin and fresh material taken from the four compartments of a cow's stomach were incubated at 38° for from 5 to 8 days and the methoxyl group determination was made on the material at the beginning and at the end of the experiments. The results obtained are recorded in Table V.

It will be observed that a loss of the methoxyl group took place in all our experiments, the greatest loss occurring when the material from the first compartment of the stomach was used.

The controls (Experiments 6 and 7) show that the loss of the methoxyl group obtained in the previous experiments is due to the

TABLE VI.
Digestion Experiments at 38° with Stomach Juice (First Pouch). Incubation Period 7 Days.

Experi- ment No.	Material added.	Amount of lignin added.	Methoxyl added in form of lignin.	Methoxyl found after in- cubation	Loss of methoxyl.
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>
16	Cellulose (filter paper).	0 3032	0 0425	0.0380	10.6
17	“ “	0 3080	0.0431	0.0411	4.6
18	Asbestos.	0 3045	0 0426	0.0371	12.9

methoxyl group added in the form of free lignin and not to the combined lignin present in the stomach material. The slight loss of the methoxyl group obtained in the controls is negligible and well within the experimental error. Our results also indicate that the addition of toluene does not check the demethoxylation process.

We also conducted similar experiments, using fresh material taken from the large and small intestine of a cow, and as our results indicate no loss of the methoxyl group, we, therefore, omit this experimental data.

Experiments were carried out in like manner with the fresh stomach juice taken from the first compartment of a cow's stomach, but no loss of the methoxyl group in the lignin was observed even when the pH of the medium was varied from 2.0

to 8.4. We found, however, if the lignin was first mixed with cellulose (filter paper) and this made into a paste with stomach juice that a loss of the methoxyl group was obtained (Table VI). The cellulose, however, was not a specific for this reaction, as a loss of the methoxyl group was obtained when asbestos was used in place of the cellulose. The explanation for this phenomenon would appear to be that the aerobic conditions (such as obtained in the experiments when cellulose or asbestos was used) favor the removal of the methoxyl group from, or the degradation of the lignin. In the experiments in which stomach juice only was used anaerobic conditions prevailed almost entirely.

We wish to express our thanks to Dr. F. W. Miller, Senior Veterinarian, and Superintendent T. E. Woodward, both of the Bureau of Dairy Industry, for their kind cooperation.

SUMMARY.

1. Lignin, prepared from corn cobs by the alkali method was fed to herbivorous animals (cows) and carnivorous animals (dogs). An increase in the benzoic acid (hippuric acid) eliminated in the urine was observed when lignin was ingested.

2. The methoxyl group determinations made on the lignin fed and that eliminated in the feces indicate that lignin suffers a loss of the methoxyl group in passing through the animal body and hence is broken down by the animal organism.

3. Experiments conducted *in vitro* indicate that the demethoxylation or degradation of free lignin takes place in the stomach of the animal, and is not brought about by bacteria but rather by some other agent, possibly an enzyme present in the gastric juice of the animal.

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THE CHEMISTRY OF THE LIPOIDS OF TUBERCLE BACILLI.

VI. CONCERNING TUBERCULOSTEARIC ACID AND PHTHIOIC ACID FROM THE ACETONE-SOLUBLE FAT.*

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INTRODUCTION.

In the analysis of the acetone-soluble fat isolated from the human type of tubercle bacilli, Strain H-37, we found, as described in Paper V (1), that the liquid fatty acids consisted of a small percentage of linoleic acid together with a large amount of a peculiar saturated fatty acid which was an oily liquid at room temperature. The acid was optically active and it possessed the important biological property of stimulating the proliferation of monocytes and epithelioid cells, and the subcutaneous injection of the substance into normal healthy animals leads to the formation of massive tubercular tissue.¹

Liquid saturated fatty acids with similar chemical, physical, and biological (2) properties have been isolated from every fraction of the lipoids of the tubercle bacilli. The first acid of this series was discovered in the phosphatide and has been described under the name of phthioic acid (3), and we have later found analogous acids in the wax fractions (4). It is evident, therefore, that liquid saturated fatty acids of high molecular weight that are optically

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† Holder of the Milton Campbell Research Fellowship at Yale University, 1928-29.

¹ The biological experiments have been carried out at The Rockefeller Institute for Medical Research and the results will be published shortly by Drs. Sabin, Doan, and Forkner.

active are constant and peculiar constituents of the lipoids derived from tubercle bacilli, and it is equally evident from the results obtained in Dr. Sabin's laboratory that the biological activity of the lipid fractions is associated with the optically active fatty acids.

In the present investigation we had about 50 gm. of the crude fatty acid in our possession and it was therefore of peculiar interest to subject it to a more thorough examination than has been possible with the small amounts that have been available previously. For the purpose of purification it was found advantageous to prepare the methyl ester of the acid, and the ester was subjected to fractional distillation in high vacuum in a specially constructed apparatus. Two principal fractions, the boiling points varying by about 50°, were obtained.

The lower boiling fraction yielded on saponification a liquid saturated fatty acid which was isomeric with stearic acid, $C_{15}H_{36}O_2$, and which we designate by the name tuberculostearic acid. It is optically inactive and it possesses no biological activity comparable to that of phthioic acid. It is interesting, however, as has been shown in preliminary experiments, that the sodium salt of tuberculostearic acid possesses a bactericidal action against *Bacillus lepræ*.² In this respect as well as in its physical properties tuberculostearic acid is similar to the hexa- and octadecanoic acids that have been synthesized by Stanley, Jay, and Adams (5).

The higher boiling fraction gave an acid which was a white solid at room temperature and which melted at 28°. The acid was found to be isomeric with cerotic acid, $C_{26}H_{52}O_2$, and it is optically active; $[\alpha]_D = +7.98^\circ$. The biological activity of the acid, as reported from Dr. Sabin's laboratory, is comparable to that of the phthioic acid isolated from the phosphatide.

Inasmuch as all of the optically active liquid saturated fatty acids occurring in the lipoids of tubercle bacilli have been found to be biologically active, we are inclined to correlate the biological effect as dependent upon or associated with the optically active fractions of the fatty acids. We regard, therefore, the acid described above as the purest specimen of phthioic acid so far obtained.

² These experiments have been conducted by Professor W. L. Kulp of the Department of Bacteriology, Yale University, and will be reported elsewhere.

That the optically active acids themselves possess these peculiar biological properties cannot be asserted definitely at this time. Further investigation must determine whether the biological activity is due to the action of a chemical compound such as a peculiarly constituted optically active fatty acid or whether it is caused by the presence of minute impurities.

The work of Goris (6) indicated that liquid saturated fatty acids were present in the lipoids of tubercle bacilli, but he believed that they consisted of caproic and possibly caprylic acids together with other unidentified acids. So far as we are aware this is the first time that any products similar to tuberculostearic acid and phthioic acid have been prepared in a reasonably pure condition.

In conclusion, for reasons to be stated later, we believe that we are dealing with a series of new fatty acids. While only two members of this series have been isolated up to the present time, it seems probable, when a sufficient quantity of material will be available for investigation, that other similar acids will be discovered.

EXPERIMENTAL.

Distillation of the Free Acids.

The crude liquid saturated fatty acid had been isolated as described in a former paper (1) and it formed a rather dark yellowish oil that weighed 49.1 gm.

In a preliminary experiment about 15 gm. of the free acids were distilled under reduced pressure but the results were not entirely satisfactory. Two fractions of distillate were obtained and a considerable amount of thick, dark colored non-volatile residue remained in the flask. The first fraction was a light yellow oil with a low optical activity, $[\alpha]_D^{20} = +1.33^\circ$. The second fraction was a thick yellow oil that solidified at about 18° and the specific optical rotation was $+6.84^\circ$. It seemed probable from these results that the crude liquid acids consisted of a mixture of optically active and inactive acids. For the more thorough separation of these products the methyl esters were prepared and fractionated as described below.

Preparation of the Methyl Esters.

The remaining crude liquid acids, 34 gm., were united with 4.4 gm. of the second fraction of the distillate, mentioned above, the mixture was dissolved in 500 cc. of absolute methyl alcohol containing dry hydrochloric acid, and the solution refluxed for 9.5 hours, when the esters separated as an insoluble oil. The solution was concentrated by distillation, diluted with water, extracted with

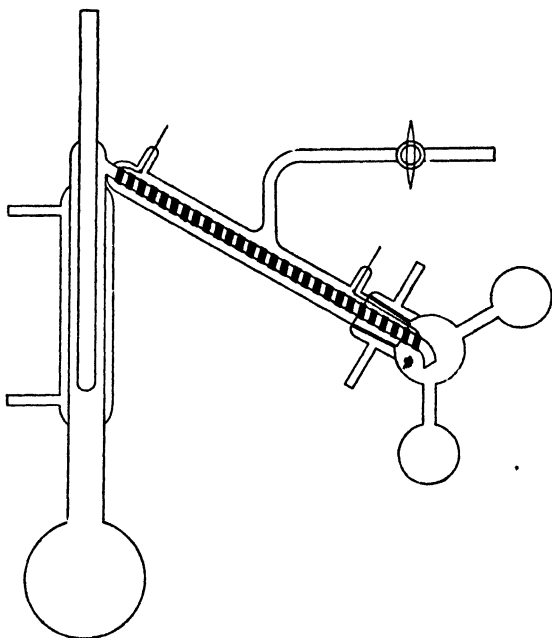


FIG. 1. All-glass apparatus for distillation.

ether, and the ethereal solution was washed first with water and later with a dilute solution of sodium carbonate. The alkaline solution turned purple in color and the extractions with dilute sodium carbonate were continued until the washings were colorless. The ethereal solution was dried with sodium sulfate, filtered, and the ether was distilled. The residue, containing the crude esters, formed a dark brown oil, and after drying in a vacuum desiccator it weighed 37 gm.

The distillations were conducted in the all-glass apparatus which is shown in Fig. 1.

*Apparatus for Fractional Distillation in Very High Vacuum.*³

The apparatus shown in Fig. 1 was connected with a Hyvac oil pump through a two-stage mercury diffusion pump. The vacuum was estimated with a 300 cc. MacLeod gage which had a 1 mm. capillary. Before the distillation was started, the vacuum system was carefully tested, and it was usually possible to get the mercury in the gage to click against the end of the capillary or to come within a couple of mm. from it. This corresponds to a pressure of about 10^{-5} mm. If this was not the case, the leak was located with an electric vacuum tester and then sealed up.

The substance to be distilled was first put into a 125 cc. round bottom flask. This flask was then sealed on to the fractionating column of the apparatus. In the middle of the column was a tube of thin glass. The inside diameter of the tube was just slightly larger than that of the thermometer used. Around the outlet tube from the fractionating column was wound a nichrome band. The band could be heated electrically to keep the outlet tube warm if the distillate showed any tendency to solidify. The mantle around the outlet tube was connected with the receiving flasks for the distillate through a water-cooled ground joint. This joint made it possible to use a larger number of receiving flasks. For our purposes only four were used. They were placed symmetrically to each other at 90° angles. When the temperature during the distillation started to show rapid changes, one of the receivers was fused off and the ground joint turned 90° to bring another receiving flask in line with the outlet tube.

When the apparatus had been used for a distillation it was cut down and the distilling flask taken off. After careful cleaning of all parts and putting on new receivers to replace those that had been sealed off, a new distilling flask with the next substance to be distilled was fused on and the apparatus again connected with the vacuum line.

³ We are indebted to Dr. Gösta Åkerlöf for designing this apparatus and assisting in its operation.

Distillation of the Methyl Esters.

The distilling flask after being sealed onto the condenser was heated by means of an air bath, the temperature being kept between 320–330°. The boiling point rose slowly from 140–260°. At the beginning the pressure was about 0.0005 mm., but as the temperature rose the vacuum decreased and at the end was about 2 mm. The distillate was a yellow oil which weighed 26 gm. It

TABLE I.
First Fractionation of Esters.

Fraction No.	Temperature of air bath.	Boiling point.	Description.
	°C.	°C.	
1	160–170	140–148	Mobile yellow oil.
2	180–190	150–175	" " "
3	210–240	185–230	Thick yellow oil, solidified at 10°.
4	240–300	235–245	" " " " " 10°.

TABLE II.
Second Fractionation of Esters.

Fraction No.	Temperature of air bath.	Boiling point.	Description.
	°C.	°C.	
1	170–190	140–145	7.6 gm. mobile yellow oil.
2	190–200	145–160	3.6 " " " "
3*	220	170	Few drops of " "
4	250–260	Mostly at 200 and at 0.001 mm.	7.8 gm. thick " "

* Fractions 3 and 4 were added to the residue in the distillation flask and fractionation was continued.

solidified when cooled in ice water. The residue in the distillation flask was a thick dark oil weighing about 11 gm., which did not distil when the temperature of the air bath was raised to 360°.

The distilled esters were fractionated, with the same apparatus, and a pressure of 0.0002 to 0.0005 mm. was maintained throughout the operation. The fractions shown in Table I were collected. Only a few drops of a thick dark oil remained in the distillation flask.

Fractions 1 and 2 were united and redistilled as shown in Table II. The pressure was from 0.0005 to 0.0002 mm.

Examination of the Ester, Fraction 1.

The substance was optically inactive. 0.7769 gm. of substance made up to 10 cc. with ether gave no rotation. When 0.1559 gm. of substance was digested with 25 cc. of Hanus' solution for 30 minutes, there was no absorption of iodine; hence the ester was saturated.

0.1224 gm. substance: 0.1394 gm. H_2O and 0.3422 gm. CO_2 .

Found. C 76.26, H 12.74.

Calculated for $C_{18}H_{36}O_2CH_3(298)$. " 76.50, " 12.76.

The analytical results indicate that the substance is an optically inactive ester of a saturated fatty acid which is isomeric with stearic acid.

Saponification of the Ester, Fraction 1.

The remainder of the ester, 7.3 gm., was saponified by refluxing with 3 per cent alcoholic potassium hydroxide for 2 hours. About one-half of the alcohol was distilled off and the remaining solution was diluted with water. In order to remove any unchanged ester the alkaline solution was extracted with ether. After washing and filtering, the ethereal solution was evaporated to dryness but only a few mg. of residue were obtained. The soap solution was acidified with hydrochloric acid and the fatty acid was extracted with ether. The ethereal solution was washed with water, treated with norit, filtered, and the ether was distilled. A faintly yellow oil remained which, after drying in a vacuum desiccator, weighed 6.5 gm. The acid solidified when cooled in ice water and when warmed gradually it liquefied at 14–15°. The acid was optically inactive. 0.7513 gm. of substance in 10 cc. of alcohol showed no rotation in a 1 dm. tube.

For analysis the substance was dried at 61° *in vacuo* over dehydrite, but there was no loss in weight.

0.1455 gm. substance: 0.1658 gm. H_2O and 0.4046 gm. CO_2 .

Found. C 75.84, H 12.75.

Calculated for $C_{18}H_{36}O_2(284)$. " 76.05, " 12.67.

Titration.—0.3550 gm. of substance dissolved in neutral alcohol, with phenolphthalein as indicator, required 12.38 cc. of 0.1 N alcoholic KOH.

Found.	Mol. wt. 286.
Calculated for $C_{18}H_{36}O_2$.	" " 284.

The silver salt was prepared by adding an alcoholic solution of silver nitrate to the alcoholic solution of the potassium salt. The silver salt, separating as a white amorphous precipitate, was filtered, washed with dilute alcohol and alcohol, and dried *in vacuo* over sulfuric acid. The silver salt was practically insoluble in hot alcohol, ether, or benzene.

0.3099 gm. substance: 0.0854 gm. Ag.

Found.	Ag 27.55.
Calculated for $C_{18}H_{36}O_2Ag$ (390.88).	" " 27.60.

The composition of the free acid and of the silver salt as well as the molecular weight agree with the formula $C_{18}H_{36}O_2$. The acid is therefore isomeric with stearic acid. In order to indicate the origin of this acid we propose to designate it by the name tuberculo-stearic acid.

Examination of the Ester, Fraction 4.

Fraction 4 of the ester distilled at 200° at a pressure of about 0.001 mm., and it was a thick yellow oil that solidified when cooled to 10° . That the substance was saturated was proved by the fact that no iodine was absorbed when a sample was tested by the Hanus method.

Rotation.—0.7735 gm. of substance dissolved in ether at 20° and made up to 10 cc. gave a reading in a 1 dm. tube of $+0.68^\circ$; hence $[\alpha]_D^{20} = +8.79^\circ$.

0.1432 gm. substance: 0.1666 gm. H_2O and 0.4109 gm. CO_2 .

Found.	C 78.26, H 13.01.
Calculated for $C_{27}H_{54}O_2$ (410).	" 79.02, " 13.17.

Saponification of the Ester, Fraction 4. Isolation of Phthioic Acid.

The remaining portion of the ester was saponified and the free acid was isolated as described under tuberculo-stearic acid. The

new acid was obtained as a faintly yellowish thick oil which solidified on cooling to room temperature and it melted at 22–23°. After drying in a vacuum desiccator it weighed 7.4 gm.

For purification the acid was dissolved in 20 cc. of acetone and the solution was cooled in ice water, when a dense white powder separated which did not show any definite crystalline structure. The precipitate was filtered on a cooled Buchner funnel, washed with ice-cold acetone, and dried *in vacuo*. It formed a snow-white soft solid and weighed 3 gm. Heated in a capillary tube, the acid melted at 28° and solidified at 26°.

For analysis the substance was dried at 61° *in vacuo* over dehydrite. but there was no loss in weight.

0.1091 gm. substance: 0.1266 gm. H₂O and 0.3158 gm. CO₂.

Found. C 78.94, H 12.98.

Calculated for C₂₆H₅₂O₂(396). " 78.78, " 13.13.

Titration.—0.3465 and 0.5558 gm. of substance dissolved in neutral alcohol, with phenolphthalein as indicator, required 8.64 cc. and 13.93 cc. of 0.1 N alcoholic KOH.

Found. Mol. wt. 401, 399.

Calculated for C₂₆H₅₂O₂. " " 396.

Rotation.—0.4414 gm. substance dissolved in ether and made up to 10 cc. gave in a 1 dm. tube a reading of +0.338°; hence $[\alpha]_D^{25} = +7.65^\circ$. 0.5940 gm. of substance dissolved in alcohol and made up to 10 cc. gave in a 1 dm. tube a reading of +0.44°; hence $[\alpha]_D^{25} = +7.40^\circ$.

The acid was probably not quite pure but the analysis and molecular weight agree fairly well with the formula C₂₆H₅₂O₂.

The potassium salt of the above acid was readily soluble in alcohol, but on cooling the solution in ice water a snow-white amorphous precipitate separated which dissolved on warming the solution to room temperature. For further purification the acid was dissolved in alcohol, neutralized to phenolphthalein with 0.1 N alcoholic potassium hydroxide, and the solution, after being concentrated to 60 cc., was cooled in ice water. The precipitate was filtered, washed with cold alcohol, redissolved in warm alcohol, and again precipitated by cooling. After this operation had been repeated a third time, the alcoholic solution of the purified potas-

sium salt was precipitated by adding an alcoholic solution of barium acetate. The barium salt was a white amorphous powder which was very slightly soluble in ether or in cold alcohol. It was more soluble in hot alcohol, but on cooling the solution the salt separated as an amorphous precipitate. Since the barium salt could not be crystallized, it was decomposed by shaking with ether and dilute hydrochloric acid. The ethereal solution was washed with water until it was free from hydrochloric acid. It was then dried with sodium sulfate, filtered, and the ether was distilled. The residue was a colorless oil when warm and a snow-white solid when cooled to room temperature. It melted at 28° and solidified at $26-27^{\circ}$.

For analysis the acid was dried at 61° *in vacuo* over dehydrite.

0.1170 gm. substance: 0.1352 gm. H_2O , and 0.3364 gm. CO_2 .

Found. C 78.41, H 12.93.

Calculated for $C_{26}H_{52}O_2$ (396). " 78.78, " 13.13.

Titration.—0.3963 gm. of substance dissolved in neutral alcohol, with phenolphthalein as indicator, required 9.94 cc. of 0.1 N alcoholic KOH.

Found.

Mol. wt. 398.

Calculated for $C_{26}H_{52}O_2$. " " 396.

Rotation.—0.4400 gm. of substance dissolved in alcohol and made up to 10 cc. gave in a 1 dm. tube a reading of $+0.351^{\circ}$; hence $[\alpha]_D^{25} = +7.98^{\circ}$.

The silver salt was prepared by adding an alcoholic solution of silver nitrate to an alcoholic solution of the potassium salt. The white amorphous precipitate was filtered, thoroughly washed with alcohol, and dried *in vacuo* over sulfuric acid. The substance was a white amorphous powder insoluble in alcohol, ether, benzene, or in water. When heated in a capillary tube, it turned slightly brown at 55° , became transparent at 57° and fused to a black liquid at $162-164^{\circ}$.

For analysis the silver salt was dried at 61° *in vacuo* over dehydrite. There was no loss in weight but the color darkened and the particles appeared to be transparent.

0.2486 gm. substance: 0.0532 gm. Ag.

Found.

Ag 21.40.

Calculated for $C_{26}H_{51}O_2Ag$ (502.88). " 21.45.

The analytical values recorded above show that the substance is a saturated fatty acid and that it is an isomer of cerotic acid, $C_{26}H_{52}O_2$. The low melting point and the optical activity must depend upon a peculiar constitutional configuration.

As will be reported elsewhere by Drs. Sabin, Doan, and Forkner, the acid possesses important biological properties. In order to indicate the biological activity and the relation of the substance to tuberculosis, we propose to designate this acid by the name phthioic acid. At the present time we are inclined to regard the liquid phthioic acid obtained from the phosphatide as consisting of a mixture of the solid optically active phthioic acid and the liquid optically inactive tuberculostearic acid which are described in this paper.

Other Fractions of Liquid Saturated Fatty Acids.

We isolated from the alcoholic mother liquors containing the more soluble portion of the potassium salt an acid which was liquid at room temperature. The substance differed from tuberculostearic acid and also from phthioic acid in that the molecular weight as determined by titration was 413 and the specific optical rotation was $+8.83^\circ$.

The non-volatile residue from the first distillation of the esters yielded on saponification a thick, dark colored oil which on standing at room temperature deposited a small amount of a solid substance.

Lack of time and material has prevented a complete examination of these various fractions. The facts noted above point to a probability, however, that the original liquid saturated fatty acids contained other acids besides those described in this paper.

SUMMARY.

A partial examination has been made of the liquid saturated fatty acids occurring in the acetone-soluble fat obtained from tubercle bacilli.

By fractional distillation of the methyl esters two principal fractions were isolated consisting of two new fatty acids.

The lowest boiling fraction has been named tuberculostearic acid. Its composition corresponds to the formula $C_{18}H_{36}O_2$ and it is therefore isomeric with stearic acid. Tuberculostearic acid

is liquid at room temperature and it is optically and biologically inactive.

The higher boiling fraction contained an acid which is solid at ordinary room temperature, melting at 28° , and optically active, $[\alpha]_D^{20} = +7.98^{\circ}$, which has been named phthioic acid. Its composition corresponds to the formula $C_{26}H_{52}O_2$ and it is therefore isomeric with cerotic acid.

The biologically active constituents of the fat are associated with the optically active phthioic acid.

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THE EFFECT OF THE DIET OF THE HEN ON THE IRON AND COPPER CONTENT OF THE EGG.*

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The total iron content of an egg of average size is about 0.8 to 1.0 mg. of Fe. Both the poultryman and the consumer are interested in the possibility of increasing the amount of iron above this figure.

The poultryman is devoting his efforts to the production of eggs which contain the maximum amounts of the essential elements and compounds needed for the development of strong and sturdy chicks. Much work has been done on the variations in the amounts of the organic entities in eggs, but the variation in the amounts of the minor inorganic elements has been given but little attention.

Upon investigating the iron present in the egg and the chick, it is found that the margin between the amount present in the egg and the quantity needed to produce the hemoglobin in the chick is very small. The iron content of a day old chick, determined by actual analysis or by calculation of the amount in the hemoglobin of a newly born chick, is between 0.6 and 0.7 mg. These figures show that very little iron can be wasted during the development of the egg into the chick and that the chick has practically no store

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of iron from which additional hemoglobin can be built. This fact is further demonstrated by the rapidity with which anemia develops when day old chicks are placed on a diet very low in iron such as milk and rice (1). An increased iron content of the egg may be a factor in the hatchability of the egg and the production of more vigorous chicks.

The increasing use of eggs in the human dietary necessitates a more complete knowledge of the variation in the amounts of the different essential components of the egg. Because of the low iron content of milk, many pediatricians today advocate the incorporation of fresh egg yolk into the diet of infants. Any increase in the iron content of egg yolk therefore would be of direct value in infant feeding.

The work that has been done on the effect of diet on the iron content of the egg is of conflicting nature and it is difficult to decide which of the published data are correct. Sherman states (2) "whether the iron content of eggs can be increased by giving to poultry food rich in iron is a disputed question."

The demonstration of the importance of copper in nutrition is so recent that little attention has been given to its distribution in food materials, while practically no work has been done on the possibility of increasing the amount present in some of our important foods. Since the two elements are so closely related in the process of hemoglobin building, we felt it was also important to study the copper content of the eggs as well as the iron content.

Fleurent and Levi (3) have reported 20 mg. of copper per kilo of moisture-free egg yolks. McHargue (4) found only 6 mg. per kilo in the yolk and practically none in the whites. Recent work by Lindow, Elvehjem, and Peterson (5) gives the copper content of egg yolk as 8 mg. per kilo of dry matter. It is important therefore to verify the normal copper content of eggs as well as to determine the variation due to the diet of the hen.

With these questions in mind, we made the following study of the effect of the hen's ration on the iron and copper content of the egg yolk and the copper content of the egg whites.

EXPERIMENTAL.

Thirty pullets were divided into three groups of ten each. They were all fed a basal ration of scratch feed consisting of 2 parts of

yellow corn and 1 part of whole wheat; a mash made of yellow corn, ground wheat, ground oats, wheat middlings, wheat bran, and meat scraps; oyster shell; and whole milk. All the hens were irradiated 10 minutes daily. Group I received the basal ration

TABLE I.
Weekly Egg Production of Individual Hens.

Hen No.	Ration	Week 11.	Week 12.	Week 13.	Week 14.	Total.
		No. of eggs.				
651	Basal.		4	1	3	8
655			5	2	1	8
656		1	3	2		6
657		2	4	5	3	14
658		1	5	5	2	13
659		3	3	4	2	12
660		3	4	1	3	11
661			2	4		6
662			3	2	1	6
Total.		10	33	26	15	84
725	Basal + Fe.			2	2	4
726		3	3	4		10
727				1	3	4
729		3	3	1		7
730					2	2
731					1	1
732		4	4		3	11
Total.		10	10	8	11	39
737	Basal + Fe and Cu.	2	2	5	1	10
739					1	1
741		2			3	5
742					1	1
744		7	5	5	2	19
746			5	4		9
Total		11	12	14	8	45

only; Group II was fed the basal ration plus an iron supplement equivalent to 50 mg. of Fe per hen daily; Group III received the basal ration, the iron supplement, and copper equivalent to 0.5 mg. of Cu per hen per day. The iron was added as a solution of

ferric sulfate and the copper as a solution of copper sulfate to the milk each day. The hens were kept in pens equipped with trap nests in order to obtain individual records.

The eggs used for iron and copper analysis were produced during the 11th, 12th, 13th, and 14th weeks after the experiment was started. The individual egg production of the hens is given in Table I.

All the eggs were kept in an ice box from the time of production until they were prepared for analysis. They were then boiled until hard, the shell removed, and the white and the yolk separated very carefully. The whites and the yolks from the eggs produced by one hen during 1 week were placed in separate dishes, weighed and dried for moisture determinations. The variation in the moisture content of the different samples was so small that we will not tabulate the figures for the entire group of samples. The average moisture content for all the samples was 48.9 per cent for the yolks and 87.7 per cent for the whites.

Methods of Analysis.—Because egg yolks are high in phosphorus, difficulty due to the fading of the colored ferric sulfocyanate was encountered when the Thomson method was used for the determination of iron. The modified method (6) reported by Elvehjem and Hart was applicable but rather long for the analysis of so many samples. We found that by treating the ash with an oxidizing agent such as nitric acid the amount of fading was greatly reduced. The products formed on heating the nitric acid solution, however, often affected the color development. We, therefore, used 30 per cent H_2O_2 for the oxidation of the ash and obtained much better results. Fowweather (7) used hydrogen peroxide (perhydrol) for the destruction of the organic matter in place of ashing, but we found that merely oxidizing the ash with H_2O_2 after ignition in a furnace gave very satisfactory results. A slight amount of fading was noted in a few samples even when H_2O_2 was used. This difficulty was eliminated by extracting the colored ferric sulfocyanate with amyl alcohol, a procedure which Kennedy (8) found to be very satisfactory.

The complete method used is outlined as follows: A sample of egg yolk (1 gm.) was ashed, the ash taken up in 3 cc. of concentrated HCl acid, 3 to 4 drops of 30 per cent H_2O_2 added to the solution of the ash and heated until the liberation of oxygen

ceased. The solution was diluted, filtered, and made up to a volume of 50 cc. A 10 cc. aliquot was placed in a 50 cc. stoppered cylinder to which 10 cc. of amyl alcohol (measured accurately) and 5 cc. of 20 per cent KCNS were added, and the solution shaken

TABLE II.
Iron Content of Egg Yolks.

Hen No.	Ration.	Week 11.	Week 12.	Week 13.	Week 14.
Fe in dried egg yolk.					
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
651	Basal.		0.0144	0.0142	0.0141
655			0.0141	0.0169	
656		0.0098	0.0112		0.0130
657		0.0112	0.0164	0.0174	0.0140
658		0.0115	0.0102	0.0115	0.0156
659		0.0152	0.0175	0.0162	0.0160
660		0.0179	0.0141	0.0141	0.0155
661			0.0115	0.0130	
662			0.0139	0.0176	0.0177
Average		0.0131	0.0137	0.0151	0.0151
725	Basal + Fe.			0.0140	0.0128
726		0.0132	0.0148	0.0165	
727					0.0138
729		0.0135	0.0123		
730					0.0113
731					0.0140
732		0.0174	0.0147		0.0150
Average		0.0147	0.0139	0.0152	0.0134
737	Basal + Fe + Cu	0.0135	0.0131	0.0152	0.0132
739					0.0180
741		0.0139			0.0115
742					0.0167
744		0.0159	0.0145	0.0140	
746			0.0148	0.0127	
Average		0.0144	0.0141	0.0140	0.0148

thoroughly to insure complete extraction of the color. The colored layer of amyl alcohol was transferred to a colorimeter cup and the amount of iron present determined by comparison with the color of a standard solution prepared in the same manner.

The iron content of the egg white is so low that iron determinations were not made on that part of the egg.

The copper content of the egg yolks and the egg whites was

TABLE III.
Copper Content of Egg Yolks.

Hen No.	Ration.	Week 11.	Week 12.	Week 13.	Week 14.
		Cu in dried egg yolks.			
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
651	Basal.		0 00074	0 00085	
655			0 00065	0.00060	
656		0 00099	0 00090		
657		0.00080	0.00090	0.00068	0.00063
658		0.00098	0.00063	0.00077	0.00077
659		0 00080	0.00074	0.00066	0.00071
660		0.00080	0.00082	0 00074	0.00084
661			0 00065	0.00067	
662		0.00070	0.00066	0.00062	
Average....		0.00087	0 00075	0.00070	0.00071
725	Basal + Fe.			0 00062	0 00060
726		0.00082	0 00092	0.00078	
727					0.00066
729		0.00081	0 00070	0 00076	
730					0.00060
731					0.00067
732		0.00080	0 00086		0.00060
Average ..		0 00081	0.00083	0.00072	0.00063
737	Basal + Fe + Cu.	0 00097	0 00082	0.00069	0 00068
739					0 00060
741		0 00090			0 00078
742					0 00066
744		0.00083	0.00088	0 00072	
746				0.00068	
Average.....		0.00090	0.00083	0.00070	0 00068

determined by the Biazzo method as outlined by Elvehjem and Lindow (9).

The iron content of the egg yolks from the eggs produced by the hens in the different groups is given in Table II. The average of all the figures is 0.0143 per cent. It is seen that the majority of

the figures do not vary a great deal from this figure. The eggs from the hens receiving iron and copper were no higher in iron than those from the hens on the basal ration.

TABLE IV.
Copper Content of Egg Whites.

Hen No.	Ration.	Week 11.	Week 12.	Week 13.	Week 14.
		Cu in dried egg whites.			
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
651	Basal.		0.00052	.	0 00038
655			0 00057	0.00054	
656		0 00066	0.00049		0.00049
657		0.00057	0 00066	0.00053	0.00049
658		0 00051	0 00054	0.00057	0.00053
659		0 00061	0.00056	0.00059	0.00043
660		0 00040	0 00050	0.00072	0.00063
661			0 00058	0 00054	
662		0 00057	0.00055	0 00069	
Average.		0 00055	0 00056	0 00058	0 00051
725	Basal + Fe.			0.00074	0 00041
726		0 00062	0 00058	0.00055	
727					0 00041
729		0.00070	0.00056	0.00064	
730					0.00069
731					0.00041
732		0.00053	0 00068		0.00075
Average.. . . .		0 00062	0 00061	0.00064	0.00053
737	Basal + Fe + Cu.	0 00058	0 00055	0 00056	0 00040
739					0 00062
741		0.00050			0 00038
742		.			0 00043
744		0 00063	0.00054	0 00052	
746			0 00059	0.00057	
Average.		0.00054	0.00056	0.00055	0.00046

The copper content of the egg yolks is given in Table III and the copper content of the egg whites is given in Table IV. The average of the figures for the quantity of copper in the yolks is 0.00076 per cent and for the whites 0.00056. It is interesting to ob-

serve that the egg albumin is practically free from iron but it contains almost as much copper as does the egg yolk. The figures also show that the copper content of the yolk and the white is not influenced by feeding either iron alone or iron together with copper to the hen at the levels used in this work.

CONCLUSIONS.

The average iron content of egg yolk is 0.0143 per cent of Fe.

The quantity of iron in the egg yolk cannot be increased by feeding the hen daily 50 mg. of iron or by feeding 50 mg. of iron plus 0.5 mg. of copper.

The average copper content of egg yolk is 0.00076 per cent and of egg white is 0.00056 per cent. The amount of copper in the yolk and in the white is not increased by feeding the hen daily 50 mg. of iron or 50 mg. of iron plus 0.5 mg. of copper.

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THE ESTIMATION OF GLYCOGEN IN SMALL AMOUNTS OF TISSUE.

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Studies on the glycogen content of biologic material have been handicapped by the fact that the more reliable chemical methods for analysis necessitate the use of considerable amounts of tissue. As a consequence, experiments which involve the use of small animals, such as tadpoles, or the isolation of a small specific tissue, such as a branch of a nerve or even repeated determinations of the glycogen content of an organ under observation can be carried out only by using procedures designed for macro estimations.

The micro estimation of glycogen has been confined, in the main, to histologic staining procedures which in my experience and in that of many others have been found to yield contradictory and erroneous results. The so called specific histologic reaction of glycogen has been employed by Mayer (8), Vastarini-Cresi (20, 21), Policard and Noël (15), and Roques (16). They have made use of such substances as cresolfuchsin, benzidene, iodine, resorcinol, fuchsin, rosaniline, and ammonium carminate. Much physiologic evidence has been submitted, based on the glycogen content of tissues analyzed by histochemical procedures, and in many instances it has been at variance with the results obtained by chemical analysis (17).

Since the early work of Brücke (11), Külz (7), Pfüger (9, 10), and Pfüger and Nerking (14), refinements in the technique of their analytic procedures for the estimation of glycogen have been made by numerous investigators, but at the present time the most satisfactory procedure, it seems to me, is the method of Pfüger (12, 13). The refinements proposed for the Pfüger method include the precipitation of the albumin by mercury

(1, 2), the use of the centrifuge in place of filtration, the removal of proteins by precipitation with trichloroacetic acid (4, 6), and the use of colloidal iron for the removal of albumins (18).

It has been definitely established that 60 per cent potassium hydroxide at 100° does not destroy glycogen, that glycogen is quantitatively precipitated from a 70 per cent solution of alcohol, and that the optimal condition for its conversion to glucose is in 2.2 per cent hydrochloric acid at 100° (5). Following the conversion of glycogen to glucose, one has recourse to any of the several titrimetric or colorimetric procedures for the estimation of the glucose so obtained.

Slosse (19), making use of the principles of Pflüger's method, developed a technique by which he was able to determine glycogen in amounts of tissue of the order of magnitude of 500 mg.

It would seem that a technique could be developed by which glycogen could be estimated chemically in amounts of tissue of the order of 5 to 15 mg., and that the principles for the isolation of glycogen from biologic material, as outlined by Pflüger, could be adapted to minute amounts of tissue, provided there was a rigorous adherence to microchemical manipulations. In determining on a method for the estimation of glucose following the acid inversion of the isolated glycogen, the new procedure outlined by Folin (3) has given extreme satisfaction and is now employed as a routine. The several notes which Folin has presented in a later paper, in the main, have been borne out by my early experiments and those difficulties eliminated previous to the publication by Folin.

Essentially the method presented here involves only the well known principle of the Pflüger procedures for the isolation of glycogen and the determination of glucose by the Folin ferricyanide method. Only the details of technique which have been found essential in handling minute amounts of tissue and of the apparatus by which glycogen can be isolated and converted into glucose, form the contribution of this paper.

A brief description of the apparatus is pertinent. In Fig. 1, the glass-stoppered Tube A, 4 × 110 mm., is used as a weighing tube and serves for the digestion of the tissue with alkali and for the purification of the glycogen by precipitation with alcohol and washing. Pipette B serves for the removal of the super-

natant alcohol and washings from the precipitated glycogen without disturbing the precipitate. Pipette C is used, following the inversion of the glycogen to glucose, to transfer the solution

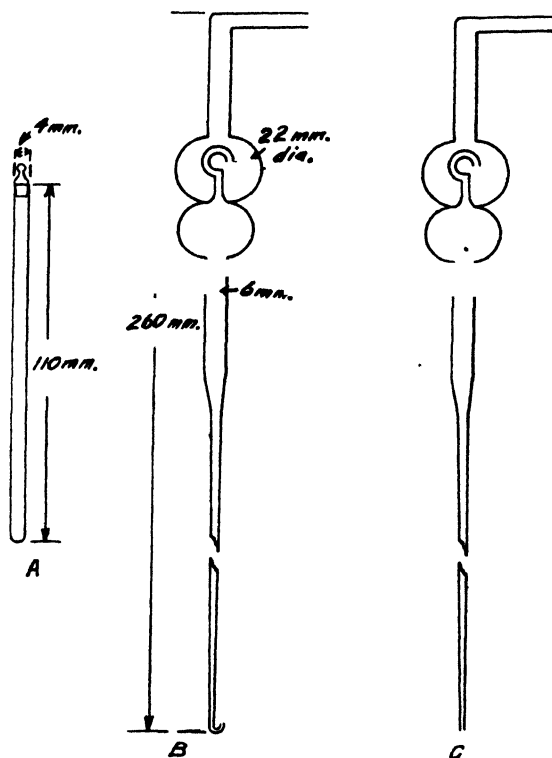


FIG. 1. Apparatus for the isolation of glycogen and its conversion into glucose. Tube A, the glass-stoppered weighing tube for the digestion of the tissue with alkali and the purification of glycogen by precipitation with alcohol and washing; Pipette B, the pipette for the removal of the supernatant alcohol and washings from the precipitated glycogen; Pipette C, the pipette used for transfer of the glucose solution and washings from the digestion tube to the tubes graduated at 25 cc.

of glucose and the washings from the digestion tube to tubes graduated at 25 cc.

A piece of tissue weighing 5 to 15 mg. is placed in a tared glass-stoppered tube, as illustrated in Fig. 1, and which contains 0.1

cc. of 60 per cent potassium hydroxide. With experience, one can estimate easily the weight of material and only a few seconds of the time elapse between the removal of material from the animal and its immersion in concentrated alkali. Hence, the danger of glycogenolysis is reduced to a minimum. With postmortem specimens it is not necessary to weigh the material in a tared tube, but the specimens may be weighed directly on a balance and then transferred to a tube containing concentrated alkali. This procedure obviously is not so accurate but has given satisfaction for most types of work. The isolation of the glycogen is accomplished by precipitation with alcohol in the same digestion tubes. Inversion by means of acid is also carried on without transfer. The removal of the various solutions for washing the glycogen is accomplished by means of a pipette possessing a curved tip (Pipette B, Fig. 1), and the transfer of the acid inversion solution to tubes for the estimation of the glucose is done by the use of a straight pipette (Pipette C, Fig. 1). The estimation of the glucose according to the technique of Folin is done in Pyrex tubes of 25 cc. capacity.

Technique.

A sample of the tissue of 5 to 15 mg. weighed either directly, or indirectly by difference in the weight of the tube with and without the sample, is digested for 1 to 2 hours, depending on the type of tissue, in 0.1 cc. of 60 per cent potassium hydroxide on a steam bath. Fragile material, such as liver, requires much less time for hydrolysis than firm cardiac muscle. The tube should be shaken occasionally to insure complete digestion of the protein material. At the end of this time the glycogen is precipitated by the addition of 0.1 cc. of water followed by 0.6 cc. of 95 per cent alcohol and 0.1 cc. of 1 per cent aqueous sodium sulfate solution. The purpose of the sodium sulfate is to carry down mechanically the precipitated glycogen along with the precipitate of sodium sulfate. The solution is allowed to stand for several hours, or better overnight, to insure completion of precipitation, after which time it is centrifugalized. The supernatant liquid is removed by means of the special pipette and the precipitate is washed successively with 70 per cent alcohol, 95 per cent alcohol, absolute alcohol, and petroleum ether in portions of 0.3-cc.

Mixing of the precipitate with these several solutions is accomplished by inverting the tube or by agitation with a fine stream of air, using the capillary Pipette C (Fig. 1). The tubes are centrifugalized and the supernatant liquid is removed with the special pipette. After the final washing, the remaining liquid is removed by heating for a short time on the steam bath. To hydrolyze the isolated glycogen, 0.2 cc. of water and 0.3 cc. of normal hydrochloric acid are added; the solution is well mixed by a current of air blown through the solution by means of the small glass capillary tube, and hydrolysis is carried out by heating on a steam bath for 3 hours.

Estimation of Glucose.

As previously mentioned, this is accomplished by the ferricyanide method which Folin has described for capillary blood. The hydrolysate is transferred to a measuring flask or cylinder by use of Pipette C (Fig. 1), the tube is washed by filling it twice with water, and the washings are combined with the original hydrolysate. An aliquot portion of this solution is then taken for the determination of glucose. The best results are obtained with a sample which contains from 0.03 to 0.05 mg. of glucose. The volume of the aliquot portion to be used can be arrived at only by trial, that is, by making the volume of the sample, for example, to 10 cc. and using 5 cc. for the estimation of glucose. If this determination demonstrates a greater content of glucose than 0.03 to 0.05 mg. a smaller sample can be taken. In the analysis of such tissue as liver, 0.5 to 1 cc. of the 10 cc. volume is adequate, whereas certain cardiac muscle may require the total of the hydrolysate in order to obtain a sufficient amount of glucose for estimation.

The aliquot portion used is diluted with water to a volume of approximately 4 cc., and is neutralized by the addition of 5 N sodium hydroxide added in drops. If the total amount of hydrolysate is used, this will require 3 drops, and aliquot portions will require a corresponding proportion of alkali. It is important that the solution be rendered not too alkaline with sodium hydroxide, and the amount of 5 N sodium hydroxide in excess should never exceed more than a fraction of 1 drop. We have not found this degree of alkalinity to be detrimental to the esti-

mation of glucose by this method. 1 cc. of the sodium carbonate-sodium cyanide solution and 1 cc. of the potassium ferricyanide solution of Folin are then added and the tubes heated on the steam bath for 8 minutes. It has been my experience that heating for 10 to 12 minutes is not detrimental. However, oxidation is complete at the end of 8 minutes. The tubes are cooled and 3 cc. of the Folin ferric sulfate solution in gum acacia are added. 10 minutes are allowed for the development of color, at the end of which time the volume is diluted to 25 cc. These colors are compared with a standard prepared, according to Folin, from 3 cc. of a solution of glucose containing 0.01 mg. of glucose in each cc., or what is more convenient, a glucose standard is prepared in a like manner to Folin's so that 1 cc. is equivalent to 0.01 mg. of glycogen. This solution can be obtained by preparing a glucose standard containing 0.0093 mg. of glucose in each cc. The percentage of glycogen is calculated from the formula:

$$\frac{\frac{0.03 \times 20}{R} \times A}{W} \times 100 = \text{per cent of glycogen}$$

where R equals the reading of the unknown, A is the fraction of the hydrolysate used for the estimation of glucose, and W is the weight of the sample.

Results.

Tables I to V, incorporating results which were obtained by this method, demonstrate that glycogen may be recovered from solutions with a maximal error of 5 per cent when subjected to the several steps of the procedure, and that duplicate analyses of a tissue give reproducible results.

The data presented in Table I show that glycogen in aqueous solutions is inverted to glucose and gives a reducing power equivalent to 93 per cent of its weight of glucose; also, that the same equivalent of reducing power is obtained with a maximal loss of 3 per cent when glycogen is recovered by the addition of alcohol to an aqueous solution and the precipitate is washed with alcohol in strengths of 70 per cent or greater.

The effect of potassium hydroxide at room temperature and at 100° on the recovery of glycogen from solutions is shown in

Table II. The maximal amount of glycogen which failed to be recovered in these two groups of determinations is 5 per cent of the quantity used. This indicates the reliability of the procedure when applied to solutions of pure glycogen.

That the presence of protein material does not introduce an element making for a greater error of recovery is shown in Table III. The recovery of glycogen is not hindered by the presence of gelatin.

TABLE I.
Recovery of Glycogen from Solutions of Glycogen.

	Tube No.	Colorimeter readings.*	Glycogen recovered.	Glycogen recovered.
		mm.	mg.	per cent
Group 1.†	1	20	0.020	100
	2	20	0.020	100
	3	20.2	0.0198	99
	4	19.5	0.0205	102
	5	20	0.020	100
	6	20.3	0.0198	99
Group 2.‡	1	20.1	0.0199	99.5
	2	20.1	0.0199	99.5
	3	20.6	0.0194	97.0

* Glucose standard 0.0186 mg. set at 20 mm.

† Six tubes, each containing 0.2 cc. of solution of glycogen (0.02 mg.) were hydrolyzed by heating for 3 hours at 100°. Glucose was then determined by the regular procedure.

‡ The glycogen in three tubes, each of which contained 0.02 mg. of glycogen, was recovered by precipitation from water with alcohol, washing the precipitate, and hydrolyzing with hydrochloric acid.

In Table IV are given the results of a series of determinations of glycogen in the liver of a rabbit together with a second series of determinations to prove that added glycogen may be recovered with only slight loss from such a tissue. In estimating the glycogen content of a tissue such as liver, the use of small samples may lead to erroneous results, due to the fact that the sample does not represent an average portion of the tissue. This is caused by the presence of a large duct or vessel which may be incorporated in certain samples and not in others. For this

TABLE II.

*Recovery of Glycogen from Solutions of Potassium Hydroxide, 60 Per Cent, Cold and Hot.**

	Tube No.	Colorimeter readings.	Glycogen recovered.	Glycogen recovered.
		<i>mm.</i>	<i>mg.</i>	<i>per cent</i>
Group 1.†	1	20.0	0.020	100
	2	20.6	0.0194	97
	3	20.1	0.0199	99.5
	4	20.3	0.0197	98.5
Group 2.‡	1	20.8	0.0192	96
	2	21.0	0.0190	95
	3	21.0	0.0190	95
	4	21.0	0.0190	95
	5	20.7	0.0193	96
	6	20.8	0.0192	96
	7	20.2	0.0198	99

* Glucose standard 0.0186 mg. set at 20 mm.

† In the first group, each tube, containing 0.02 mg. of glycogen in 0.1 cc. of potassium hydroxide 60 per cent was treated with 0.1 cc. of water and 0.4 cc. of alcohol, was allowed to stand 3 hours, centrifugalized, washed, and hydrolyzed.

‡ In the second group, the tubes containing 0.02 mg. of glycogen in potassium hydroxide 60 per cent were heated for 3 hours at 100°, and then treated as described for the first group.

TABLE III.

*Recovery of Glycogen Added to Protein Material (Gelatin) Which Did Not Contain a Measurable Amount of Glycogen.**

Tube No.	Weight of gelatin.	Colorimeter readings	Glycogen recovered.	Glycogen recovered.
	<i>mg.</i>	<i>mm.</i>	<i>mg.</i>	<i>per cent</i>
1	11.1	20.8	0.0192	96
2	8.0	21.0	0.0190	95
3	9.7	20.0	0.0200	100
4	12.5	19.2	0.0208	104

* Standard 0.0186 mg. glucose set at 20 mm. To each of five tubes containing variable amounts of gelatin was added 0.02 mg. of glycogen contained in 0.1 cc. solution of potassium hydroxide 60 per cent. These were heated at 100° for 3 hours and treated as in the regular procedure.

reason, an average value obtained from several determinations gives a truer picture.

TABLE IV.
*Recovery of Glycogen from Tissue of Rabbit.**

Direct estimation of glycogen content of liver tissue.			Estimation of glycogen content of tissue from same liver, to each sample of which had been added 0.24 mg. glycogen and this value subtracted from total mg. glycogen recovered.		
Tube No.	Weight of sample.	Glycogen found.	Tube No.	Weight of sample.	Glycogen found after subtracting 0.24 mg. glycogen.
	mg.	per cent		mg.	per cent
1	11.6	3.96	1	20	4.11
2	15.0	3.54	2	16	3.56
3	17.7	4.23	3	14.5	3.68
4	11.8	3.67	4	15.7	4.21
Average.....		3.85			3.89

* Glucose standard 0.0279 mg. set at 20 mm.

TABLE V.
Duplicate Analyses of Several Tissues for the Glycogen Content.

Tissue.	Tube No.	Weight of sample.	Glycogen.
		mg.	per cent
Human heart.	1	17.3	0.16
	2	13.9	0.14
	3	11.8	0.16
	4	10.0	0.15
Bundle of His, human heart.	1	9.8	0.17
Bundle of His, horse heart.	1	9.3	0.71
	2	11.0	0.71
	3	9.8	0.70
Human liver.	1	13.0	3.90
	2	10.2	4.19
	3	6.0	3.95
	4	9.2	4.04

The results presented in Table IV show what may be accomplished in the recovery of a quantity of glycogen equivalent in

magnitude to that contained in the tissue to which it is added. The average result of the determination of glycogen content of the tissue is 3.85 per cent, whereas the average value for glycogen content of the same tissue, to which glycogen had been added and this quantity subtracted from the total amount recovered, was 3.89 per cent.

In Table V are presented representative analyses of tissue from the liver and from the heart to show the high degree of reproducibility of results which may be obtained on tissues of widely varying texture and glycogen content.

DISCUSSION.

It is obvious that such a method as that given here is subject to many possibilities of error unless one is at all times cognizant of the exact technique to which one must adhere. It is probably superfluous to reiterate the precautions necessary to be taken, but perhaps a short description of the several steps liable to error in the analytic procedure will not be amiss.

The details of weighing the sample depend on the conditions of the experiment. If direct weighing is used, the sample can be transferred to the tube and immersed in the strong alkali by means of a glass rod without danger of error due to loss of any portions of the sample. If indirect weighing is employed it is important that the alkali be placed in the bottom of the digestion tube by delivery from a burette having a long, fine tip, so that the sides of the digestion tube are not wet by the solution. If this precaution is taken, the sample can be immersed in the alkali with the aid of a nickel or glass rod without danger of altering the weight of the tared digestion tube. Care must be taken in the removal of the supernatant liquid from the precipitated glycogen not to disturb the precipitate when pipetting off the supernatant liquid. This is readily accomplished by using the special pipettes as illustrated. These pipettes are constructed in the manner shown, in order to obviate the possibility of any saliva contaminating the solution.

The inversion of the glycogen presents no difficulty, and it is only necessary to insure thorough mixing of the precipitate with the solution of hydrochloric acid. The Folin ferricyanide method for estimating the glucose has been highly satisfactory,

and it is well to be conversant with the precautions which Folin has mentioned in his later work on his method. It is imperative that the solution be not too alkaline, as stated.

I have not experienced difficulty in the use of gum acacia as a protective colloid, providing the gum acacia is reasonably fresh and moldy growth has not developed in it. The solutions have kept well for approximately a month. It has been the custom to prepare daily the dilute standard glucose solution from the stock standard because of the apparent deterioration of the dilute glucose standard.

It is not recommended that a micro technique such as that described in this paper should be employed when tissue is available in larger quantities, because in selecting samples of material of such small weights, a representative and average tissue is not obtained. However, in the analysis of a particular type of tissue of which only a small amount may be available, this technique proves of advantage. Interest in determining the percentage of glycogen in such tissue as the bundle of His in man and animals, and the glycogen content of organs of very small animals, caused me to develop this micro method.

SUMMARY.

By means of the principles of Pflüger's method for the estimation of glycogen in biologic material, namely the destruction of the tissue by means of concentrated alkali, the isolation and purification of the glycogen by means of alcoholic precipitation, followed by the inversion of the glycogen to glucose, and the estimation of the glucose so obtained, a technique has been described which enables one accurately to estimate the glycogen content of a specimen of tissue weighing from 5 to 15 mg. Glycogen, when added to such tissue, may be recovered with a degree of accuracy sufficient for purposes of studies of tissue glycogen. Data are presented on the application of this technique to the estimation of glycogen in hepatic and cardiac tissue, showing the reproducibility of the results and the satisfactory recovery of added glycogen. This technique is being employed at present in the estimation of glycogen in tissues of which only very small specimens are available.

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STEAM DISTILLATION OF THE LOWER VOLATILE FATTY ACIDS FROM A SATURATED SALT SOLUTION.

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The identification of the volatile fatty acids is most easily carried out by determining their ratio of distillation. The original direct distillation of Duclaux (1) has been largely displaced by steam distillation which, although giving constant rates, is a slow and laborious process.

It is known that butyric and propionic acids may be salted out of solution by saturation with sodium chloride or calcium chloride. It occurred to us, however, that solutions of volatile fatty acids might be steam-distilled at increased rates by saturation with some suitable salt. Because small amounts of volatile acid were to be distilled, it was found necessary to make the saturated salt solution strongly acid with a mineral acid. Magnesium sulfate (because of its cheapness) and sulfuric acid were found to be satisfactory.

Method.

The apparatus used (Fig. 1) was a slight modification of Dyer's (2). To 100 cc. of water containing 5 to 20 cc. of 0.1 N volatile fatty acid, 70 gm. of magnesium sulfate were added (at 0° the solubility of magnesium sulfate is 76.9 gm.) and 2 cc. of 50 per cent sulfuric acid. Six 100 cc. fractions were collected and titrated with 0.1 N or 0.01 N sodium hydroxide, with 1 per cent alcoholic solution of phenolphthalein as indicator. Several cc. of indicator solution should be used in titrating with 0.01 N alkali. The acids distil over very rapidly and care must be taken to distil the first and second 100 cc. fractions slowly. 10 to 15 minutes should be required to collect the first 100 cc. and 10 minutes for the second. The remaining fractions may be distilled in 6 or 7 minutes. The

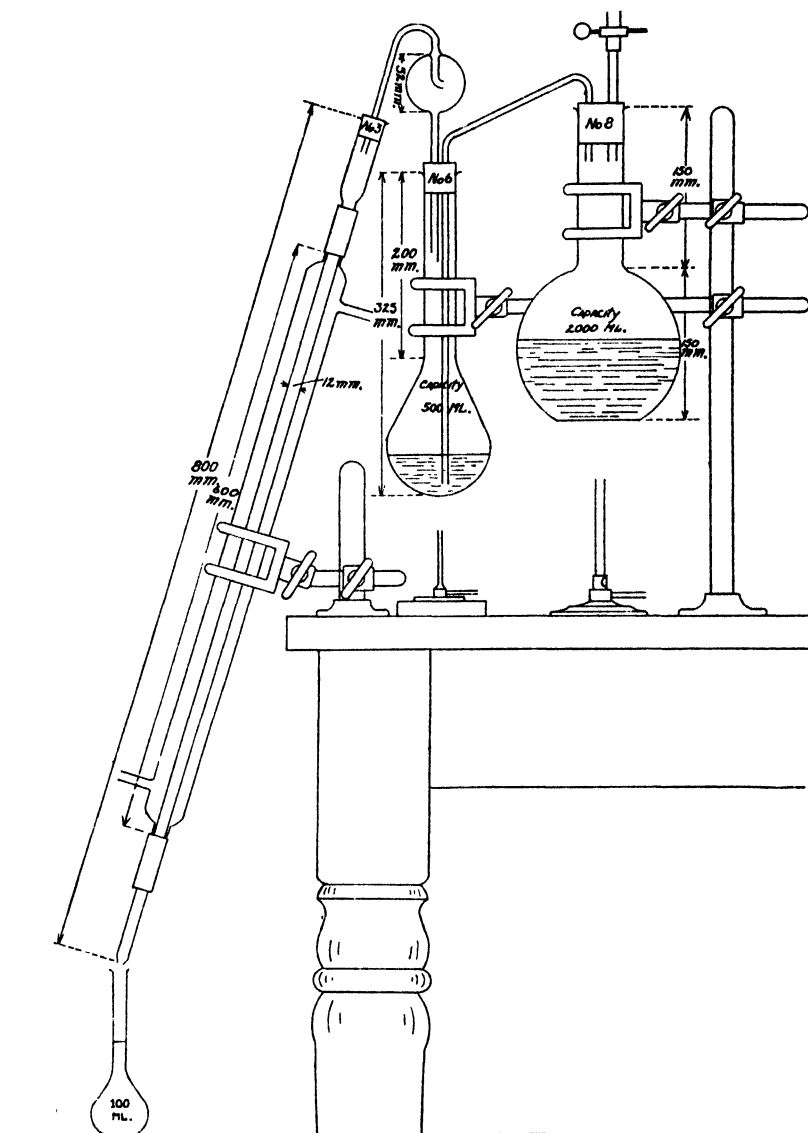


FIG. 1. A 2 liter flask acts as steam boiler. Material to be distilled is placed in a Kjeldahl flask, the volume of which is kept constant at 150 cc.

salt solution containing the fatty acid should be brought to boiling just before steam is run through. The temperature of the salt solution was found to remain at 104°. In all, six 100 cc. fractions were collected.

EXPERIMENTAL.

Blanks run on the magnesium sulfate and sulfuric acid yielded the following average values:

100 cc. fractions.	0.01 N alkali. cc.
1	1.3
2	0.6
3	0.6
4	0.6
5	0.5
6	0.5

There is some variation in these values and this accounts to some degree for the variation in distillation rates.

Distillations were made with 5, 10, 15, and 20 cc. of 0.1 N formic, acetic, propionic, and butyric acids. Dyer used 0.5 gm. of acid. In this work 3 to 12 mg. (in the case of acetic acid) were used. It was our intention to apply this method to the determination of the volatile fatty acids in stools, and we had found by experiment that these amounts of acid were available for determination. The distillation rates obtained for formic, acetic, propionic, and butyric acids are shown in Table I.

When combinations of two or more of these acids are steam-distilled, the rates of distillation obtained are in proportion to the amounts of the acids present and their known rate of distillation. Table II presents the results of the distillation of known amounts of mixed acids. The rates obtained were very close to the theoretical.

DISCUSSION.

It is apparent from data presented in Table I that under the experimental conditions the rates of distillation of these fatty acids are considerably accelerated. Table III shows a comparison between the rates obtained by steam distillation from water (Dyer) and those obtained from a salt solution. By the latter

means the acids are more rapidly distilled, so that the time-consuming method of collecting them by distillation is considerably shortened. Of importance also is its assistance in the identification of formic and acetic acids. When distilled from water, the difference in the percentage between formic and acetic acids in the first 100 cc. of distillation is 12 (Table III); when dis-

TABLE I.

Distillation of Formic, Acetic, Propionic, and Butyric Acids from a Saturated Salt Solution. Variation from the Average Rate of Distillation.

Distillate.	5 cc.	10 cc.	15 cc.	20 cc.
Formic acid 0.1 N.				
cc.	per cent	per cent	per cent	per cent
100	29 ± 2	29 ± 2	31 ± 1	31 ± 2
200	50 ± 4	51 ± 3	53 ± 2	53 ± 3 (16 distillations.)
300	65 ± 4	66 ± 3	68 ± 2	69 ± 2
Acetic acid 0.1 N.				
100	62 ± 2	62.5 ± 6	63 ± 6	64 ± 5
200	84 ± 2	84 ± 4	88 ± 4	89 ± 4 (26 distillations.)
300	92 ± 2	93 ± 2	95 ± 2	96 ± 2
Propionic acid 0.1 N.				
100	91 ± 1	88 ± 3	88 ± 1	88 ± 2
200	98 ± 2	97 ± 2	96 ± 1	96 ± 1 (20 distillations.)
300	99 ± 1	98 ± 1	97 ± 0	97 ± 1
Butyric acid 0.1 N.				
100	91.5 ± 0	92 ± 2	92 ± 2	90 ± 2
200	95.7 ± 0	96.5 ± 1	97 ± 1	96.5 ± 1 (15 distillations.)
300	97 ± 0	97 ± 5	97.5 ± 1	98 ± 1

tilled from salt solution, it is 33. For the second 100 cc. fraction this difference, with water, is 15; with salt solution, 26. By this means the accuracy of identification by distillation methods for mixtures of these two acids is doubled. The difference in distillation rates, however, between acetic, on the one hand, and butyric and propionic acids on the other, is less from salt solution than

from water. The method, therefore, is of assistance in the collection and identification of mixtures of formic and acetic acids.

TABLE II.

Distillation Rates of Mixtures of Acetic and Butyric Acids.

5 cc. 0.1 N acetic acid plus 5 cc. 0.1 N butyric acid.

Per cent acid in first 100 cc. of distillate should be 77.3.

Distillations: 76.7, 77.7, 82.7, 74.7, 76.7, 76.2, 78.2; average 77.5.

5 cc. 0.1 N acetic acid plus 10 cc. 0.1 N butyric acid.

Per cent acid in first 100 cc. of distillate should be 82.

Distillations: 78.4, 82.4, 79.8, 82.8, 79.8, 82.0; average 80.8.

10 cc. 0.1 N acetic acid plus 5 cc. 0.1 N butyric acid.

Per cent acid in first 100 cc. of distillate should be 73.4.

Distillations: 68.6, 72.5, 76.8, 76.8, 73.8, 76.1, 72.4, 75.5, 71.5, 73.8; average 73.8.

Distillation Rates of Mixtures of Formic and Butyric and Formic and Acetic Acids.

10 cc. 0.1 N butyric acid plus 10 cc. 0.1 N formic acid.

Per cent acid in first 100 cc. of distillate should be 60.8.

Distillations: 61.1, 60.1.

5 cc. 0.1 N acetic acid plus 5 cc. 0.1 N formic acid.

Per cent acid in first 100 cc. of distillate should be 45.7.

Distillations: 45.2, 46.2.

TABLE III.

Steam Distillation of Volatile Fatty Acids.

The results are expressed in per cent.

Acid.	100 cc.		200 cc.		300 cc.		600 cc.	
	Dyer.	Salt solution.	Dyer.	Salt solution.	Dyer.	Salt solution.	Dyer.	Salt solution.
Formic.....	18.5	30	35.5	52	46.5	68	69.8	89
Acetic.	30.7	63	50.8	86	64.9	94	88.5	99
Propionic.	52.5	88	76.3	96	87	98	96.4	98
Butyric.....	69.9	92	90.5	97	96.0	98	98.5	98

With mixtures of three or more acids considerable help could be obtained by the comparison of the distillation rates obtained by steam distillation both from water and from salt solutions.

Table IV shows that if one had a mixture of formic, acetic, propionic, and butyric acids, the propionic and butyric acids would have been completely distilled into the first 300 cc. of distillate, the fourth and fifth 100 cc. would contain formic and acetic acids, and the sixth and seventh only formic. Thus the higher acids are rapidly eliminated in the first 300 cc. of distillate, and identification of formic and acetic acids is facilitated in the later fractions.

The variation of individual distillations from the mean is more marked with acetic than with the other acids. In our experience

TABLE IV.

*Total Acid Appearing in Each 100 Cc. Fraction of Distillate,
10 Cc. of 0.1 N Acid.*

100 cc. Fraction No.	Formic acid.	Acetic acid.	Propionic acid.	Butyric acid.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	30	62	88	92
2	22	23	9	6
3	16	9	1	0.5
4	10	3	0	0
5	6	1		
6	4.5	0		
7	2			
8	2			
9	1			

duplicate distillations are a sufficient basis for the calculation of rates. Table II shows this to be true.

SUMMARY.

1. When the lower volatile fatty acids are steam-distilled from a salt solution, characteristic rates of distillation are obtained.
2. The quantitative collection of these acids is accelerated from 30 per cent to 100 per cent.
3. The identification of formic and acetic acids is facilitated.

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A METHOD FOR THE RAPID DISTILLATION OF THE LOWER VOLATILE FATTY ACIDS FROM STOOLS.

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Methods for the accurate identification of the volatile fatty acids of the stools have long been known. Ludwig Brieger (1) in 1878 published a method, the principles of which are still in use; namely, the distillation of the acids from an acid mixture and the purification and identification of their metallic salts. Schmidt and Strasburger (2) in their well known work used the same principles. Edelstein and von Csonka (3) in 1912 and Bahrdr and Edelstein (4) in 1911 distilled stools under a partial vacuum, claiming by this technique to obtain the acids in a fairly pure state. They identified the acids by the fractionation of their silver salts. The latter method is undoubtedly very accurate but time-consuming. Hoppe-Seyler (5) after extracting the stool with alcohol, neutralized the alcoholic extract with sodium carbonate, evaporated it to dryness, took up the residue in water, acidified, and then distilled it. Hecht (6) distilled the acidified stools in steam. Cecchini (7) distilled stools directly but redistilled the first portion of distillate. These later methods are simple but open to the objection that acid-volatile products of protein and carbohydrate digestion would contaminate the distillate. The appearance of these products would be accentuated by boiling stools in the presence of strong mineral acid.

The principle we have used is the precipitation of the mass of organic material with mercuric chloride and then steam distillation of the filtrate. Before the filtrate is distilled, however, it is saturated with magnesium sulfate in order, first, to increase the rapidity of distillation and thus shorten the time-consuming

process of collecting large volumes of distillate, and, second, to obtain the maximum effect of the salting out process on the distillation rate. The distillation rate of a fatty acid will vary if it is in solution with varying amounts of any salt. Since stool filtrates do contain salts varying in concentration with each specimen, it is necessary to control this factor if the distillation rate is to be used for the identification of the acid.

Method.

Stools mixed with an equal amount of 10 per cent alkali are not only effectively deodorized but their emulsification is very greatly facilitated. If more than one stool is to be collected, a sufficient amount of alkali may be placed in a commode and the stool passed directly into the alkali. Before precipitation the alkaline stool mixture is passed through a wire sieve, such as used by the housewife. There are certain definite objections to the collection of stools in alkali. A slow hydrolysis of nitrogenous material and sugar takes place, so that after a period of a week or more there is a definite increase in organic volatile acids. In a month's time we have found an increase of 20 per cent in the volatile acids. These processes are so slow, however, that one has from 48 to 72 hours in which to work.

100 gm. of alkaline stool (50 gm. of stool) are made acid to litmus with 50 per cent sulfuric acid and diluted with 100 cc. of water. 200 cc. of 10 per cent mercuric chloride in 1.5 per cent hydrochloric acid are added. Mercury is precipitated with a thick suspension of calcium hydroxide. The latter is added until the reaction is strongly alkaline to litmus. The volume is made up to 500 cc. A Buchner funnel fitted with a piece of muslin makes rapid filtration of this bulky material possible. The first filtrate is very cloudy but the muslin is soon filled with the coarse precipitate and the filtrate becomes clear. Centrifugation in 100 cc. centrifuge tubes is also a satisfactory method. Mercuric chloride precipitation of stools yields a filtrate that is clear but of amber color, giving a faint biuret reaction. 95 per cent of the stool nitrogen as determined by Kjeldahl is removed. Of late we have been experimenting with mercuric sulfate precipitation of stools and find the filtrate to be water-clear. At this time we have no data as to the recovery of added volatile acid with the latter

method of precipitation. Excess mercury is removed by hydrogen sulfide and blown off in the usual way. No loss of volatile fatty acid occurs from running an air current at high speed through acid solutions of such concentration as we are dealing with. To 100 cc. of mercury-free filtrate, representing from 5 to 10 gm. of stool, are added 2 cc. of 50 per cent sulfuric acid and 70 gm. of magnesium sulfate. Distillation is carried out as described in the preceding paper.

Blanks were run on materials including the alkali used for collection. The blanks on each of six fractions of distillate

TABLE I.

Comparison of Total Acid Appearing in Each Fraction of Distillate When Acids Are Distilled from (A) 100 Cc. Watery Solution of Acids Plus 70 Gm. of Magnesium Sulfate; (B) 100 Cc. Blank Filtrate Plus Acids Plus 70 Gm. of Magnesium Sulfate.

100 cc. distilled, Fraction No.	Formic acid, 10 cc. 0.1 N.		Acetic acid, 10 cc. 0.1 N.		Butyric acid, 10 cc. 0.1 N.	
	A	B	A	B	A	B
	cc.	cc.	cc.	cc.	cc.	cc.
1	3.0	3.3	6.2	6.6	9.2	8.8
2	2.2	2.3	2.3	2.16	0.6	0.7
3	1.6	1.6	0.9	0.7	0.0	0.2
4	1.0	1.0	0.3	0.2		0.08
5	0.6	0.55	0.1	0.1		0.03
6	0.45	0.46	0.0	0.05		0.02

(0.24, 0.13, 0.06, 0.06, 0.06, 0.06 cc.) totaled 0.6 cc. of 0.1 N alkali. These blanks are not large enough to interfere with the accuracy of determining rates of distillation.

The purpose of distilling from a solution of magnesium sulfate was to minimize the effects of salt concentrations in stool filtrates. To test out this point, blank filtrates were prepared, starting with 100 cc. of 10 per cent alkali and to this filtrate was added formic, acetic, or butyric acid. Table I shows that the rates of distillation under the conditions of our method are comparable to the rates of distillation of pure acids distilled from a saturated solution of magnesium sulfate.

Recovery of Lower Volatile Acids Added to Stools.

Table II shows the recovery of added volatile acid to stools. The accuracy of the method appears to be ± 5 per cent. A well known method for the determination of formic acid depends upon the reduction of mercuric chloride to the mercurous salt. The recovery of added formic acid in this method is due to the fact that mercuric chloride is reduced by formic acid very slowly and with long continued heating. We are convinced that mercuric

TABLE II.
Recovery of Volatile Fatty Acids Added to Stools.

0.1 N acid distilled from 20 gm. stool. A	0.1 N acid added to 20 gm. stool B	0.1 N acid recovered. A + B C	Acid recovered. $\frac{C - A}{B}$	Expected recovery.
cc.	cc.	cc.	per cent	per cent
13.73	4.0 Formic.	17.01	82	85
13.73	8.0 "	20.38	83	88
11.78	4.0 "	15.31	88	85
10.68	4.0 "	14.30	90	85
7.88	2.0 Acetic.	9.97	104	99
7.88	4.0 "	11.94	101	99
7.88	8.0 "	16.19	104	99
10.74	4.0 "	15.23	112	99
10.74	8.0 "	18.28	94	99
13.70	4.0 "	17.90	105	99
7.88	8.0 Butyric.	15.93	100.5	98
10.74	8.0 "	18.77	100	98
13.70	4.0 "	17.57	97	98
8.88	4.0 "	13.02	103	98
11.80	4.0 Propionic.	15.78	99	98

chloride precipitation does not result in the reduction of mercuric chloride by formic acid in the stool.

Quantity of Lower Volatile Acids Found in Stools.

The distillation rate of a mixture of two known volatile fatty acids will be in proportion to the quantity of the acids present. This fact was first pointed out by Duclaux. Gillespie and Walters (8) have shown that it is possible to calculate the amount of acids from distillation rates even when there are three or more

in the mixture. Therefore the distillation rates obtained from mixtures of volatile fatty acids in the stool will demonstrate the quantity of the individual acids present provided their identity is known.

The literature on volatile fatty acids of stools furnishes sufficient data on this point. Rubner (9) in 1883 reported 79 per cent of butyric and 20 per cent of acetic acid. Nencki (10) in 1891 found that in material obtained from an iliac fistula only acetic acid was present. Brieger's results in 1878 show that three-fourths of the acid was acetic, and that there were definite amounts of butyric acid and traces of the higher volatile fatty acids. Schmidt and Strasburger reported that butyric was the chief acid with much less of acetic and traces of formic acid present. Von Csonka and Edelstein found acetic to be the principal acid, with butyric and traces of formic acids in the mixture. Bahrdt and McLean (11) give data on stools of infants in which acetic acid predominated; lesser amounts of butyric. Fischer (12) found acetic and butyric acids; Cecchini, also. Hecht, working with infant's stools found that acetic acid predominated with butyric acid in lesser amounts and formic acid present at times and absent at others. The findings of von Csonka and Edelstein, Bahrdt and McLean, and of Fischer are to be emphasized because they identified and purified the acids by the silver salt method. In general we can say that in human stools there appear acetic, butyric, and traces of formic acids and that in only rare instances have the higher acids been found.

Table III shows the titration figures of each 100 cc. fraction of distillate and Chart 1 the percentage of total acids distilled that appear in each fraction. In this and the succeeding paper we have distilled the filtrates from 150 stools. The percentage of acid appearing in the first 100 cc. of distillate has always ranged between 68 and 77 per cent. At the end of 200 cc., 85 to 91 per cent has appeared; at the end of 300 cc., 92 to 96 per cent; at the end of 400 cc., 95 to 97 per cent; in the fifth and sixth fractions there is but a trace of acid. On comparison of these findings with the rates of distillation obtained with pure acids (Chart 1), it would appear that in the first two fractions the rate is due to the rapid distillation of butyric and acetic acids, whereas in the latter fractions the rate decreases, probably because of the presence of formic acid in small amounts.

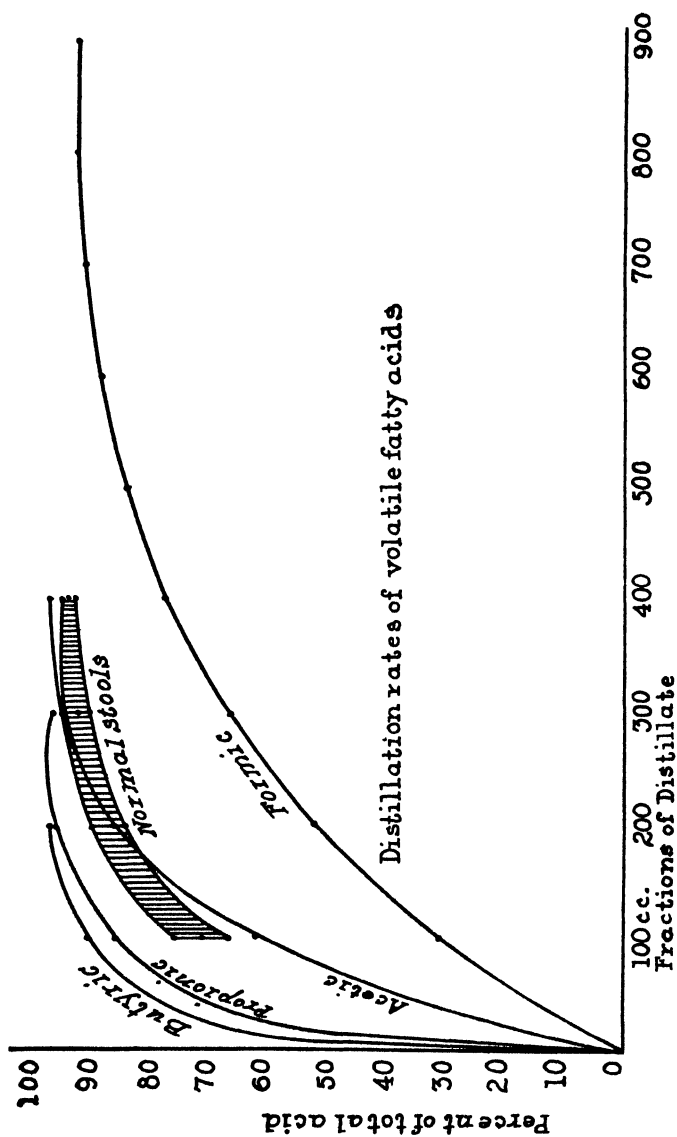


CHART 1.

In Table IV of the preceding paper it was shown that all of the butyric and acetic acids are distilled over in 400 cc. of distillate and that in the fifth fraction 6 per cent of formic acid remains. The amounts of acid found in the fifth fraction of distillate should represent formic only and since they represent 6 per cent of the total formic acid present, it is a simple matter to calculate this amount. We have done this and have found that 20 to 25 per cent of the total volatile acids is formic acid. This amount in the light of the results recorded in the literature

TABLE III.

Amount of Acid Appearing in Each of Six 100 Cc. Fractions in Normal Stools.

The results are expressed in cc. of 0.1 N acid.

Subject.	Fraction 1.	Fraction 2.	Fraction 3.	Fraction 4.	Fraction 5.	Fraction 6.	Total.	Amount stool distilled.	Total 0.1 N acid for 24 hrs.
								gm.	cc.
Fr.	8 14	1.65	0.53	0.22	0.10	0 04	10.68	6 2	145
Pl.	10 27	2 22	0 74	0.30	0.12	0 06	13 71	9.5	240
Ol.	6 25	1.64	0 64	0 27	0.11	0 04	8 95	2.9	115
Ev.	8 69	2 17	0 82	0 36	0.17	0 06	12 27	4.1	193
St.	8 72	2.14	0 76	0.28	0.15	0.06	12.11	7.0	172
Gr.	13 50	2 95	1.03	0.52	0 23	0 11	18.34	20.0	269
Ko.	13 87	2.12	0 93	0.35	0.17	0.12	17.56	4.1	143
Ad.	4 22	0.81	0 32	0.11	0.08	0 02	5 56	4 0	141
Ku.	5 82	1.18	0.47	0 22	0.14	0.10	7 93	4.4	210
Ai.	9.92	2 30	0 94	0.42	0.18	0.06	13.82	9 8	158
McC.	12 17	2 17	0.72	0 29	0 19	0.14	15.68	4.1	312
Ma.	5.27	0.97	0 28	0.09	0 04	0 03	6.68	10.0	138

seems too high. To check the amounts of formic acid in stools by another method, we determined it by the reduction of mercuric to mercurous salt. 600 cc. of distillate from a normal stool were concentrated after neutralization to a small volume and the method as employed by Shaffer and Friedemann (13) was used. Blank determinations were always run and duplicate determinations checked. Table IV shows that 6 to 15 per cent of the total volatile acid was in the form of formic acid, an average of 10 per cent. If we accept this figure as accurate, then one must conclude that in distilling these stool filtrates there appears in the distillate

a volatile acid, or acids, not of the fatty series. Every fact seems to point to these acids being present in very small amounts.

TABLE IV.
Determination of Formic Acid by Mercury Reduction Method.

Stool No.	Total acids.	Formic acid.	Per cent of total acids.
	<i>cc. 0.1 N</i>	<i>cc. 0.1 N</i>	
H68	4.59	0.43	9
G66	13.03	1.12	9
A62	11.11	1.35	12
T70	11.42	0.74	6
Q72	8.76	1.13	13
B74	6.83	0.56	8
T82	10.47	1.59	15
E86	9.04	1.20	13
A88	4.50	0.51	11
K92	7.63	0.91	12

TABLE V.
Amounts of Acetic Acid and of Butyric Acid, on the Basis That 10 Per Cent of Formic Acid Is Present in Stools.

Total acids appearing in first 100 cc. fraction.	Acetic acid.	Butyric acid.
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
68	60	30
69	56	34
70	51	39
71	48	42
72	44	46
73	41	49
74	38	52
75	35	55
76	30	60
77	27	63
78	25	65

Table V shows the percentage of acetic and butyric acids appearing in the stools for the rates of distillation that have been observed. The average distillation rate is 72 per cent in the first 100 cc. fraction, which indicates equal amounts of acetic and butyric acids.

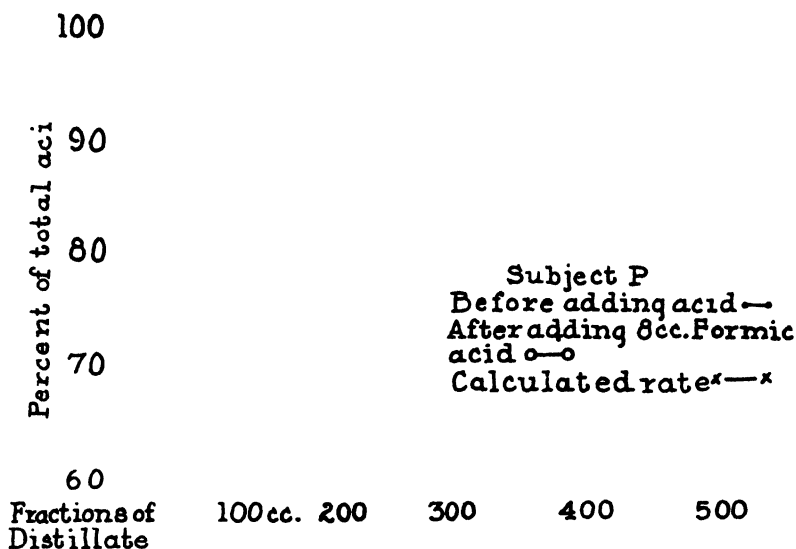


CHART 2.

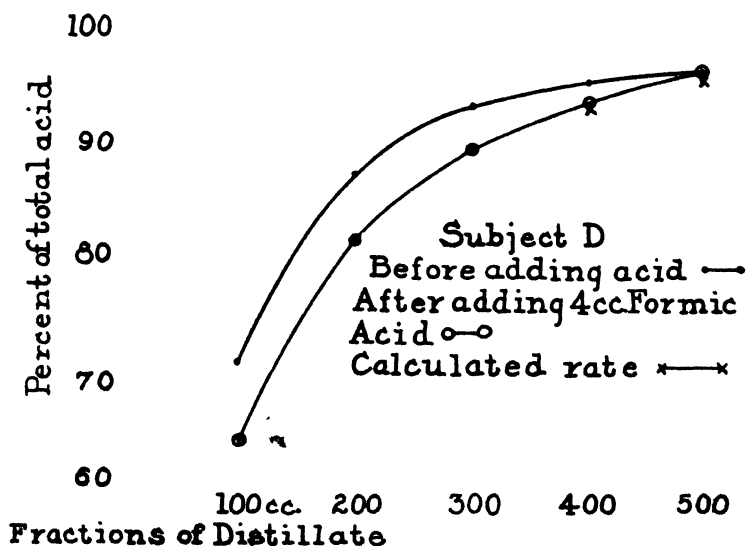


CHART 3.

If one adds to a stool a known amount of, say, formic acid in sufficient quantity to change definitely its rate of distillation, one should be able to predict the new rate of distillation. We have first calculated the acids present in a stool by the use of Table V, then added a known amount of formic or butyric acid to the same stool, and calculated the rate of distillation of this new mixture of acids by Table I presented in the preceding paper. The actual observed rate of distillation was compared with the calculated.

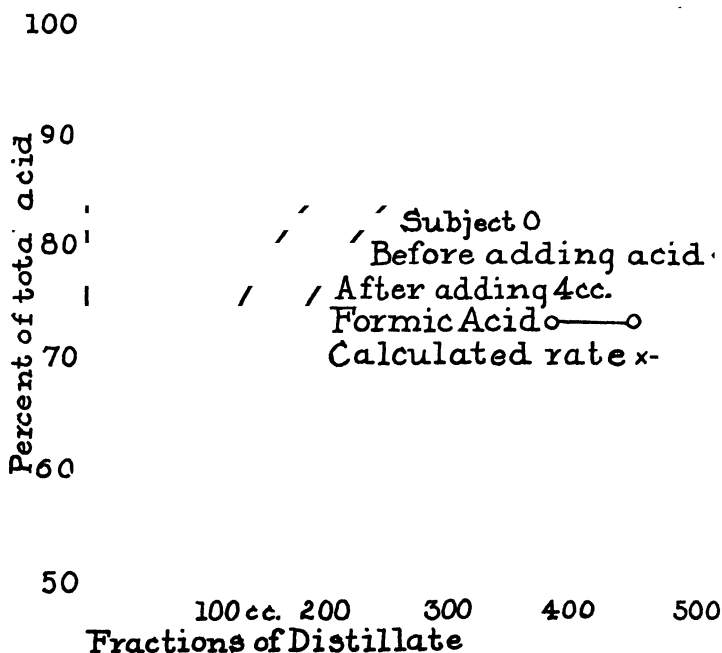


CHART 4.

The amounts of natural stool acids determined varied in the specimen from 9 to 14 cc., and the amounts of acid added, from 4 to 8 cc. Charts 2 to 6 show the rates of distillation before and after addition of a known amount of acid, as well as the calculated rates. The rather close agreement of the observed and calculated rates gives further evidence that our deductions as regards formic acid are correct, and that the amounts of non-fatty volatile acids are so small as to be negligible.

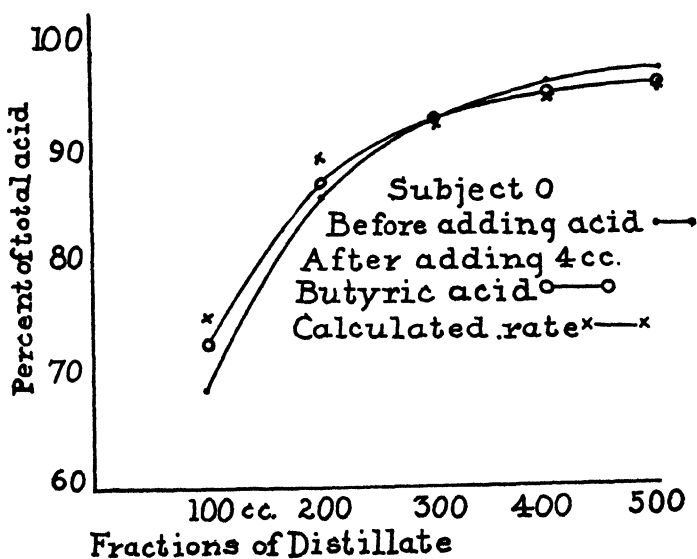


CHART 5.

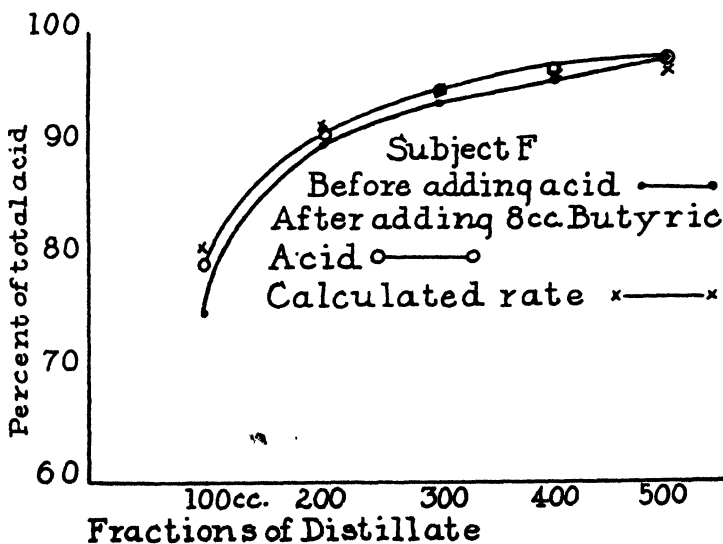


CHART 6.

CONCLUSIONS.

1. A method for the rapid distillation of the volatile fatty acids from stools is presented.
2. Volatile acids added to stools may be recovered.
3. Evidence is presented that acetic and butyric acids appear in amounts varying from 30 to 60 per cent each of the total volatile fatty acids in normal adult stools.
4. Formic acid appears in very small amounts, averaging 10 per cent of the total volatile fatty acid content.

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THE EFFECT OF DIET AND CATHARSIS ON THE LOWER VOLATILE FATTY ACIDS IN THE STOOLS OF NORMAL MEN.

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The purpose of this investigation was to collect data bearing on the amounts of food material, more particularly of carbohydrate, metabolized by intestinal bacteria. The lower volatile fatty acids are the most stable of the products of bacterial action upon carbohydrate.

By the use of the method elaborated in the preceding paper the daily determination of the lower volatile fatty acids in stools has been simplified. It was possible to follow the daily variations in the output of these acids with changes in diet. The results obtained from observing the daily elimination and concentration of these acids suggested that reabsorption from the intestine took place. If this be true, brisk catharsis should increase the elimination of the lower volatile fatty acids by a washing out process.

HISTORICAL.

We have been unable to find in the literature any report of day by day, quantitative determinations of volatile fatty acids in the stools of normal adults who were on accurately controlled diets. Up to 1910 much good work had been done showing the general nature of these acids. Brieger (1) in 1878 recognized acetic, butyric, and the higher volatile fatty acids in stool distillates. He mentions in his paper that his findings confirm those of Nencki published in the same journal in 1877. Schmidt and Strasburger (2) give Rubner's findings (1883) of butyric and acetic acids in stools. Hecht (3) in 1910 determined quantitatively by direct distillation the total volatile fatty acids in the stools of infants. He gave values from 100 to 676 cc. of 0.1 N acid per 100 gm. of stool. His highest values were observed when infants were breast-fed or when dextrin was added to the feedings. Lower values were found with high casein and fat feedings. He noted

that the output of volatile fatty acids paralleled the well being of the child. The predominating acid was acetic; butyric acid was found in smaller amounts and formic was present in traces.

Edelstein and von Csonka (4, 5) in 1912 distilled infant's stools under a partial vacuum and fractionated the silver salts of the volatile fatty acids. They found that acetic acid predominated with considerably lower amounts of butyric acid and traces of formic. In cases of intestinal catarrh the acetic and butyric acids were in equal amounts. Fischer (6) in 1913 studied the chemical constituents of stools with subjects on different kinds of diets, analyzing one representative stool very completely. He found the volatile fatty acids from 182 to 1275 cc. of 0.1 N acid for 100 gm. of dry stool. A summary of his findings is as follows:

Diet.	Cc. 0.1 N acid, 100 gm. fresh stool.
Schmidt test diet.	62-120
Protein.	50- 90
Vegetable-fruit.	53-112
Fat.	47- 94
Milk.	121

Fischer gives as the principal origin of the volatile fatty acids the fermentation of carbohydrate. He points out that the deamidation of certain amino acids in the intestinal tract (glutaminic acid) may give rise to butyric acid and that there are certain organisms which split unsaturated higher fatty acids to propionic and butyric acids. Bahrdt and coworkers (7) in a series of papers (1911 to 1914) studied experimentally the effects of all the volatile fatty acids on the peristaltic movements of the intestine of dogs, the experimental effect of volatile fatty acids on the excretion of nitrogen, minerals, and calcium, and the volatile fatty acids present in the stools of normal and diseased infants. These authors used steam distillation with a partial vacuum. The acids were identified by purification of their calcium and silver salts. 140 infant's stools were studied. In normal infants 15 to 347 cc. of 0.1 N acid were present for each 100 gm. of stool, usually over 100 cc. Breast-fed infants produced more volatile fatty acid than infants on other diets. Volatile acids in the food itself were not a source of volatile fatty acids in the stool. In sick infants it was the diet rather than the disease which influenced the output. There was no increase in volatile fatty acids in sick infants. Acetic acid formed the greater part of the acids found. Intestinal peristalsis was stimulated by the lower acids.

Roux and Goiffon (8) determined the volatile fatty acids in 1500 stools. On a mixed diet they obtained 70 to 85 cc. of 0.1 N acid per 100 gm. of stool, in cases of dyspepsia, due to excessive starch in the diet, 100 to 150 cc. of 0.1 N acid per 100 gm. of stool. With diets high in protein or fat the volatile acids were low; with diets high in cereals, the acid output was high. Cecchini, (9) studied the volatile fatty acids in a number of stools collected from patients suffering from various diseases. He directly distilled the acidulated stool, concentrated the first 50 cc. and redistilled, using the

method of Duclaux. One stool was analyzed for each type of disease and the data on seventeen stools were presented. The total volatile fatty acids found usually varied from 100 to 200 cc. of 0.1 N acid. Higher values were obtained in the group of dyspepsia with fermentation, 150 to 300 cc. Only acetic and butyric acids were found, usually a slightly greater amount of acetic acid. In the stools of the dyspepsia patients butyric acid usually predominated. The stools of this group were very bulky. Cecchini concluded that the volatile fatty acids result from bacterial fermentation of carbohydrate. In 1926 Sperry (10), working with dogs, found that 65 per cent of the volatile fatty acids of dog feces was acetic, 23 per cent butyric, and 11 per cent caproic. He criticizes Cecchini's conclusions on the ground that the rate of distillation by the Duclaux method might indicate the presence of some caproic acid as well as of butyric and acetic. It must be pointed out, however, that caproic acid has not been identified by the German workers using silver-salt fractionation.

EXPERIMENTAL.

The subjects of the feeding experiments were medical students. The food was weighed and prepared in the kitchen of the metabolism ward of Barnes Hospital. Each dietary period was of 7 days duration. The summary of each day's feeding is given in the protocols.¹ In general they may be summarized as follows:

Period.	Protein.	Fat.	Starch.	Sugar.
	gm	gm.	gm.	gm.
High protein	250	70-100	55-70	30-40
“ sugar.....	50	100	135	365
“ starch.....	50	100	400	100
“ fat.....	50-60	300	75	25
“ milk fat.	50-60	300	60	40

Carbohydrate in all fruits except bananas was estimated as sugar. Fat was milk-free except in the last period when milk fat was fed to determine the effect of butyric acid. In the period of high protein feeding 90 per cent of the protein was in the form of meat. In the high sugar period 45 per cent of sugar fed was from fruits, 55 per cent saccharose. In the high starch period 42 per cent of starch fed was from potato, 29 per cent from wheat, 21 per cent from banana. In the high fat period 50 per cent of fat was from

¹ The protocols are omitted for lack of space; data will be supplied to any one interested.—W. H. O.

corn oil, 21 per cent from meat fat, 24 per cent from nuts, and 5 per cent from eggs. In the high milk fat period 90 per cent of fat was milk fat and all the sugar given was lactose.

Stools were collected in alkali as outlined in the preceding paper. A carmin capsule was taken at the last meal in each period to demarcate the period. The lower volatile acids were determined by our method, usually on the same day or within 24 hours.

• *Results with Changes of Diet.*—Table I is a summary of the daily figures. The subjects varied markedly in the level of their 24

TABLE I.

Effect of Diet and Stool Volume on Total Volatile Fatty Acid Excretion and Concentration.

Diet.	Subject K.				Subject McC.				Subject M.			
	Average 24 hr. stool volume	Average cc 0.1 N acid in 24 hrs.	Average cc 0.1 N acid in 100 gm. stool.	Length of period	Average 24 hr. stool volume.	Average cc 0.1 N acid in 24 hrs.	Average cc 0.1 N acid in 100 gm. stool.	Length of period.	Average 24 hr. stool volume	Average cc 0.1 N acid in 24 hrs.	Average cc 0.1 N acid in 100 gm. stool.	Length of period.
	gm.			days	gm.			days	gm.			days
Regular.....	143	98	194	3	119	230	219	3	127	119	93	4
High protein ..	83	90	79	9	55	71	141	7	180	179	104	7
“ sugar. . .	222	148	159	9	205	278	139	7	329	447	134	8
“ starch. .	193	125	155	6	121	191	166	7	253	349	138	6
“ fat (no milk).....	135	147	97	8	207	178	98	7	253	239	94	7
High milk fat..	126	82	147	7	118	129	134	7	211	221	108	7

hour excretion of acids. Subject M produced almost double the amount of acid in 24 hours that Subjects K and McC produced, not because the acids were in higher concentration in his stools but because this subject's stools were twice as large as those of the other two.

Changes in 24 hour elimination of volatile fatty acids, as the result of changes in diet, followed the same general curve in all subjects. The lowest output occurred with high protein diets; it increased only slightly during high fat feeding, but was markedly increased by high sugar and starch diets. If one takes the 24

hour output during protein feeding as 100, then the changes are shown to be as follows:

Diet.	Subject K.	Subject M.	Subject McC.
Protein.	100	100	100
Sugar	164	250	391
Starch.	139	195	269
Fat...	164	133	251
Butter fat.	91	123	182

The concentration of the acids in the high protein, high fat, and high milk fat periods was of like magnitude, while in the high starch and sugar periods it was in each instance increased. The

TABLE II.

Percentage of Acid Distilled in First 100 Cc. Fraction from Stools under Varying Dietary Conditions.

The figures are averages.

Period	Subject K.	Subject McC	Subject M.	Subject O.	Subject G.
High protein.	72 6	71 6	70.4		
“ sugar.	72 5	72.6	69 1		
“ starch.	72 6	69.2	71.0		
“ fat.	74 3	72.8	70 2		
“ milk fat.	74 3	70 3	72 9		
Before catharsis.		68 1	67 3	72	72
During “		68 1	66 3	72	72

increased elimination during carbohydrate feeding was due not only to the increase in size of the stool but also to an increased production of the acids. Feeding butyric acid in the form of milk fat failed to increase the elimination of the acids and in two of the three subjects there was no increase in the concentration of the acids.

Distillation Rates.—The percentage of the total acids appearing in the first 100 cc. fraction of distillate was very nearly the same regardless of the food mixture administered (Table II). As indicated in the preceding paper the rates observed would suggest approximately 10 per cent of formic, from 60 to 40 per cent of acetic, and from 30 to 50 per cent of butyric acids.

TABLE III.

*Effect of Increasing Volume of Stool on Output of Volatile Fatty Acids,
High Carbohydrate Diet.*

Date.	Stool volume.	No. of stools in 24 hrs.	Volatile fatty acids, cc. 0.1 N solution.		Remarks.
			Per 24 hrs.	Per 100 gm.	

Subject G.

	gm.				
Feb. 19	100	1	130	130	
" 20	71	1	127	179	
" 21	102	1	186	182	
" 22	30	1	51	171	
" 23	191	1	370	192	
" 24	71	1	129	181	
" 25	157	2	242	154	MgSO ₄ , 4 gm.
" 26	301	2	331	110	" 6 "
" 27	236	2	205	117	" 10 "
" 28	714	3	479	67	" 10 "
" 29	815	4	451	55	" 30 "
Mar. 1	781	4	440	56	" 30 "
" 2	429	3	281	64	" 15 "
" 3	233	1	182	156	None.
" 4		1	182	156	

Subject O.

Feb. 22	252	1	168	134	
" 23		1	168	134	
" 24	302	1	193	128	
" 25		1	193	128	
" 26	333	1	235	142	
" 27			235	142	
" 28	265	1	204	155	
" 29			204	155	
Mar. 1	653	3	247	37	MgSO ₄ , 15 gm. 8 a.m., 3 p.m.
" 2	1140	4	410	36	" 15 " 8 " 3 "
" 3	1046	4	438	42	" 15 " 8 " 3 "
" 4	1126	4	550	48	" 15 " 8 " 3 "
" 5	149	1	155	105	None.

TABLE III—*Concluded.*

Date.	Stool volume.	No. of stools in 24 hrs.	Volatile fatty acids, cc. 0.1 N solution.		Remarks.
			Per 24 hrs.	Per 100 gm.	
Subject McC.					
Apr. 23	240	1	212	89	Diet, 50 gm. protein, 100 gm. fat, 100 gm. starch, 400 gm. sugar.
" 24	71	1	180	254	
" 25	370	1	479	129	
" 26	70	1	104	148	
" 27	Lost.	1			
" 28	53	1	89	168	
" 29	100	2	233	233	MgSO ₄ , 30 gm.
" 30	80	1	91	114	
May 1	1007	4	380	38	
" 2	887	4	427	48	
Subject M.					
Apr. 23	287	1	272	95	Diet, 50 gm. protein, 100 gm. fat, 100 gm. starch, 400 gm. sugar.
" 24	407	2	475	117	
" 25	Lost.	2			
" 26	331	1	304	92	
" 27	279	2	500	179	
" 28	217	1	269	125	
" 29	336	2	543	162	MgSO ₄ , 30 gm.
" 30	106	1	158	148	
May 1	1532	4	842	54	
" 2	870	3	450	52	

Effects of Catharsis.—The dietary experiments suggested that the lower volatile fatty acids were absorbed and that what appeared in the stools was but a fraction of the amounts produced. The fact that the subject (Subject M) with the voluminous stools eliminated the most acid pointed to this. Again, after days when no stools were passed the subsequent stools contained no additional acid. Four subjects were placed on high carbohydrate diets and following control periods of 7 days were given 30 gm. of magnesium sulfate daily (Table III). The stool volumes were increased to, in most instances, over 1000 gm., increasing the usual

output three- to fourfold. The output of acids was increased about twofold. In three of the subjects 450, 550, and 427 cc. of 0.1 N acid were maximum eliminations. Subject M, as would be expected, reached the high level of 840 cc. The distillation rates during catharsis did not change from those observed in the control period (Table II).

DISCUSSION.

The stools even on low carbohydrate diets contained hydrolyzable and fermentable sugar. In the few analyses we could perform there was found, after hydrolysis, from 0.5 to 3 per cent of sugar. The fermentation products of bacteria grown on nitrogen-containing media, containing sugar, have been shown to arise from the fermentation of the sugar rather than from amino acids (11). Our dietary experiments indicate strongly that this is true, for with high protein and minimum sugar feeding volatile acid elimination reached its lowest output.

The products of the action of *Bacillus coli* on glucose are lactic, acetic, and formic acids, alcohol, carbon dioxide, and hydrogen. In fact, they are the usual products of the catabolism of glucose produced by bacteria in general. *Bacillus welchii*, *Staphylococcus aureus*, and streptococci produce butyric acid. For the *Bacillus coli* group, 2 molecules of glucose form 2 molecules of lactic acid, 1 of acetic acid, and 1 of alcohol. Formic acid is decomposed to hydrogen and carbon dioxide by an enzyme formiase produced by the colon group of organisms.

It is a justifiable assumption that a gm. of acetic acid represents the breakdown of 6 gm. of glucose. In the preceding paper the average elimination in the stool of young men was 188 cc. of 0.1 N acid daily, of which 45 per cent was acetic acid, representing the loss of 3 gm. of glucose.

In the subjects of the dietary experiments here reported the daily losses of acetic acid in stools represented losses of glucose, as shown in Table IV.

We do not know what butyric acid as an end-product represents. It might represent lactic acid, for there is some evidence that it is formed in the case of some organisms from fermentation of lactate. We can hardly assume that it represents the breakdown of an additional amount of glucose to that calculated from acetic acid as an end-product.

The purpose of the catharsis experiments was to wash out acids that might be produced and reabsorbed. We cannot see any reason to assume that this is not the case. There may be some possibility that the increased fluidity of intestinal contents accompanying catharsis would promote bacterial fermentation. One would not expect such an explanation to account for so large an acid elimination as we have observed. The catharsis produced in these subjects lasted but 8 hours and we can only guess as to the amounts of acid which might be found if, say, a watery stool every 2 or 3 hours during the whole 24 could have been produced. The data of the catharsis experiments, if it is assumed that 45 to 60 per cent of total acid was acetic, represent the fermentation of 7 to 18 gm. of glucose, or 1 to 4 per cent of the total carbo-

TABLE IV.

Losses of Acetic Acid in Stools and the Glucose That It May Represent.

Subject.	High protein diet.			High fat diet.			High sugar diet.			High starch diet.		
	Total acid.	Acetic acid.	Glucose.	Total acid.	Acetic acid.	Glucose.	Total acid.	Acetic acid.	Glucose.	Total acid.	Acetic acid.	Glucose.
	cc	gm.	gm.	cc.	gm.	gm.	cc.	gm.	gm.	cc	gm.	gm.
K.	90	0.23	1.4	147	0.33	1.9	148	0.38	2.28	125	0.31	1.9
McC.	71	0.19	1.2	178	0.45	2.7	278	0.72	4.3	191	0.64	3.8
M.	179	0.54	3.2	239	0.73	4.4	447	1.5	9.0	349	1.0	6.0

hydrate fed. It is not unreasonable to suppose that double these amounts of sugar are daily fermented, most of the end-products being absorbed.

CONCLUSIONS.

1. Subjects on weighed diets eliminated from 60 to 300 per cent more lower volatile fatty acid on high carbohydrate than on high protein feeding.

2. Catharsis produced at least 100 per cent increase in the elimination of the lower volatile fatty acids.

3. Acetic acid elimination represents the breakdown of from 2 to 9 gm. of glucose.

4. It has been shown that 1 to 4 per cent of ingested carbohydrate is fermented by intestinal bacteria.

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THE APPARENT DISSOCIATION CONSTANTS OF TRYPTOPHANE AND OF HISTIDINE.*

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In a summary of the data dealing with the dissociation constants of the amino acids, Kirk and Schmidt (1) have pointed out that not only have the apparent dissociation constants of certain amino acids not yet been determined, but also that there is a lack of agreement in the values reported by different workers. The present report forms a part of a systematic survey of the apparent dissociation constants of the amino acids begun in this laboratory some time ago (2).

The technique employed in obtaining the titration curves and the method of calculation used were the same as those which have been described by Kirk and Schmidt (3). Tryptophane was prepared from casein according to the technique of Hopkins and Cole (4). It was recrystallized four times from dilute alcohol according to Dakin's (5) recommendation. Two preparations of histidine were used. Pfanstiehl's histidine dichloride was purified according to the method recommended by Vickery and Leavenworth (6) for crude histidine. It was recrystallized three times. A sample of free histidine prepared according to the method of Vickery and Leavenworth (7) was kindly supplied to us by Dr. H. B. Vickery. All preparations were dried over phosphorus pentoxide before use.

The titration data are shown graphically in Figs. 1 and 2. For tryptophane the following values were obtained.

$K'_a = 4.05 \times 10^{-10}$, $K'_b = 2.4 \times 10^{-12}$ and $pI = 5.89$. Simms'

* We are indebted to the Cyrus M. Warren Fund of the American Academy of Arts and Sciences for the loan of the type K potentiometer.

(8) values for tryptophane at 25° recalculated to the same basis as ours are: $K'_a = 4.25 \times 10^{-10}$, $K'_b = 1.85 \times 10^{-13}$. The agreement between the two sets of data is satisfactory. For histidine we have calculated two sets of values, one for the Pfanstiehl preparation and the other for Vickery's free base. For the former we obtained: $K'_a = 7.5 \times 10^{-10}$, $K'_{b_1} = 1.0 \times 10^{-8}$, $K'_{b_2} = 6.6 \times 10^{-13}$; for the latter the values $K'_a = 5.9$

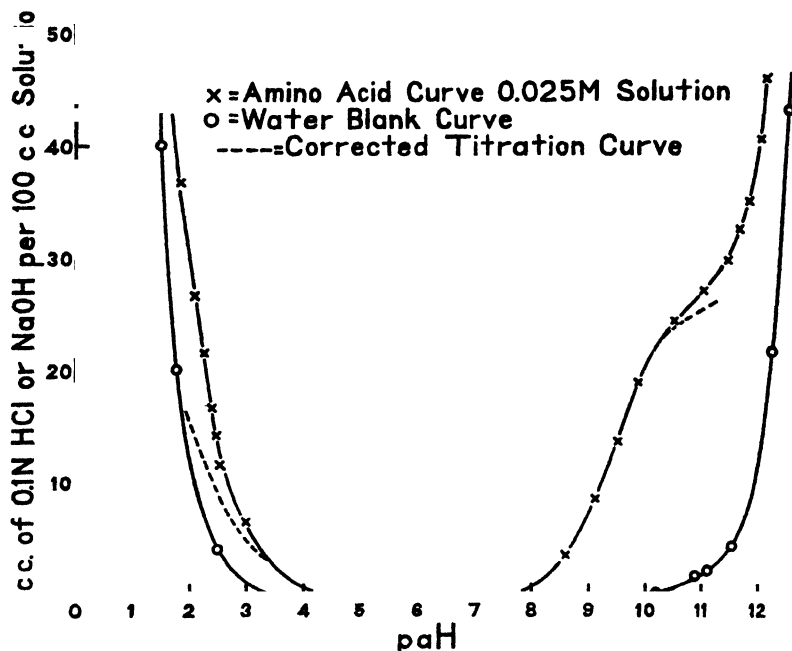


FIG. 1. Titration curves of tryptophane.

$\times 10^{-10}$ and $K'_{b_1} = 1.03 \times 10^{-8}$ were obtained. On the assumption that the average of these two sets of data represents the most probable value we have: $K'_a = 6.7 \times 10^{-10}$, $K'_{b_1} = 1.01 \times 10^{-8}$, $K'_{b_2} = 6.6 \times 10^{-13}$ and $pI = 7.6$. Simms' (8) values for histidine, recalculated to the same basis as our values, are: $K'_a = 3.89 \times 10^{-10}$, $K'_{b_1} = 1.15 \times 10^{-8}$, $K'_{b_2} = 2.90 \times 10^{-13}$ and $pI = 7.7$. The maximum deviation in our data lies in the value for K'_a . This is expected since, on account of the logarith-

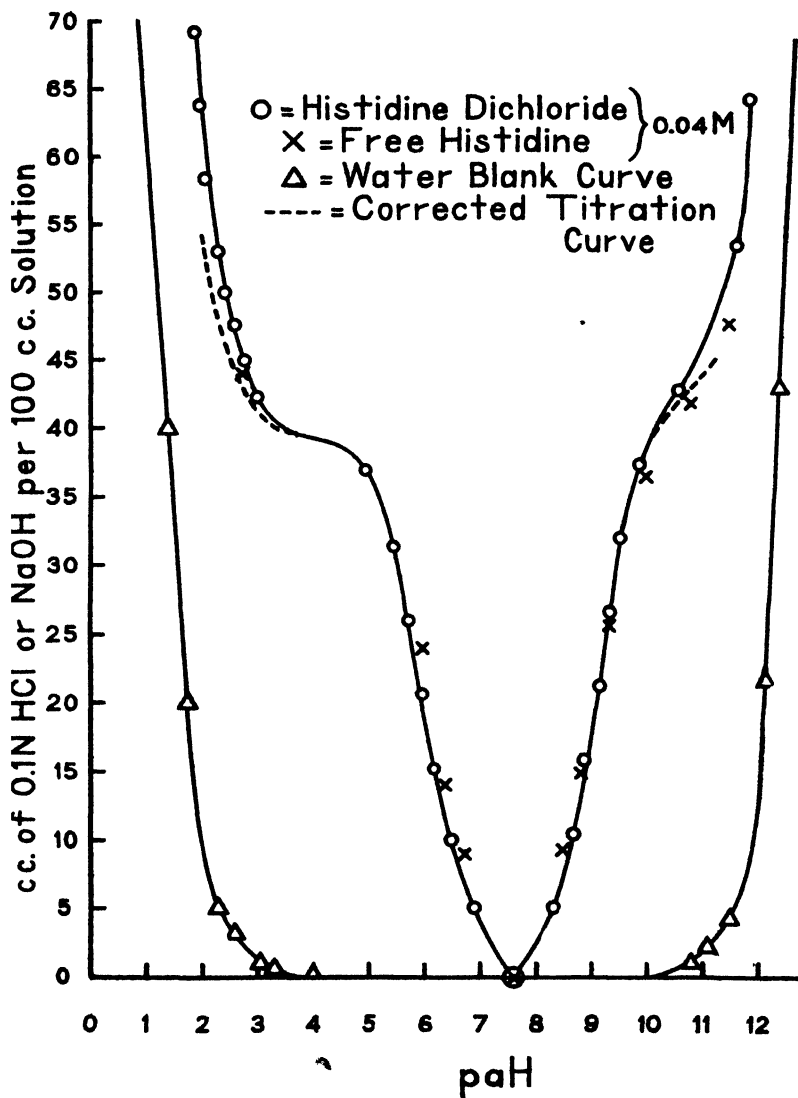


FIG. 2. Titration curves of histidine.

mic formula, small errors in this region are magnified. The value for the isoelectric point of histidine is somewhat greater than 7.2, the value which was assumed by Vickery and Leavenworth (6).

SUMMARY.

The apparent dissociation constants of tryptophane and of histidine have been determined. These data are compared with data previously published by Simms on the same subject.

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FRACTIONATION STUDIES ON PROVITAMIN D.*

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In previous studies we found that a commercial cholesterol preparation obtained from the Wilson Laboratories had a high concentration of antirachitic provitamin. In the present studies we undertook to concentrate the provitamin fraction in cholesterol and to study its properties.

175 gm. of the commercial product, Cholesterol W, were dissolved in 1200 cc. of 10 per cent solution of sodium hydroxide in alcohol and boiled under a reflux condenser for 1 hour. After chilling the solution to 38°, the mass of crystals was drained free from liquid by suction and washed with cold alcohol. The alkaline alcoholic filtrate was evaporated to dryness and extracted with boiling acetone. After evaporation of the acetone, the brown, semicrystalline mass was taken up in ether, the ether solution washed repeatedly with water and finally evaporated to dryness. The residue was still light brown and somewhat waxy in consistency. This was Fraction 1c.

The large mass of crystals separated from the alkaline alcohol above was dissolved in ether and this ether solution washed by shaking with water until the wash water was only faintly alkaline. A deep emulsion layer containing cholesterol always formed between the water and ether layers. This was separated, the cholesterol filtered off, dried, and kept as Fraction II.

157 gm. of cholesterol obtained from the original ether solution were recrystallized twice from acetone. 113 gm. were recovered. This was Cholesterol B. 50 gm. of Cholesterol B were recrystallized from acetone three additional times with a final recovery of

* These studies were in part supported by a grant from the Douglas Smith Foundation for Medical Research.

27 gm. This was Cholesterol A. The acetone mother liquors and washings from each recrystallization were evaporated to dryness and the residue kept as Fractions 1a₁, 1a₂, 1a₃, 1a₄, and 1a₅.

Sublimation suggested itself as a further method of fractionating the provitamin factor. However, since our previous experience had indicated that cholesterol is susceptible to oxidation it seemed wise to heat under reduced pressure in an atmosphere of nitrogen. To further safeguard against oxidation and moisture the N₂ was passed through linseed oil, alkaline pyrogallol, and concentrated sulfuric acid. Cholesterol A, our most highly purified cholesterol and presumably the lowest in antirachitic provitamin content, was heated slowly at a pressure of 7 to 10 mm. of nitrogen. For this purpose we used a 1½ x 8 inch Pyrex tube fitted by means of a ground glass connection at the neck with a smaller inner tube through which cold water flowed. The tip of the condenser was about 1 inch from the bottom of the outer tube so that its cooled surface caught all the sublimed cholesterol. Under these conditions a thin film of sublimate began to form on the condenser at 165°. The maximum amount of sublimate was obtained between 200° and 210°. Heating to a higher degree or continued heating after the first half hour at 210° gave no increased yield of sublimate. After 3 hours the heating was discontinued. The amount of sublimate was small, not more than 30 mg. from 500 mg. It appeared like more because it formed in a loose fluffy mass of slender, sharp, pointed plates. The unsublimed residue, after cooling, was a hard, creamy, semicrystalline mass. This was ground to a fine powder which, under the microscope, consisted of minute, flat, irregular plates. A distinct drop in the melting point of the cholesterol after heating was evidence that a chemical change of some sort occurred during heating. The melting point of Cholesterol A was 149.7° and of the residue 142.5°. The sublimate began to melt at 142° and was completely molten at 145°.

Biological Assay of Various Fractions.

In the first series of biological tests the following preparations were compared. (1) Cholesterol B, twice recrystallized from acetone; (2) Fraction II, the cholesterol which tended to form an emulsion; (3) Fraction 1c, the brown, semicrystalline material extracted from the soaps; (4) Cholesterol A, five times recrystal-

lized from acetone; (5) Cholesterol A sublimate; (6) Cholesterol A residue; (7) Fraction 1a₁, the cholesterol from the first acetone mother liquor; (8) Cholesterol W, the original commercial product.

These materials were finely powdered, spread in a thin layer, and irradiated under a quartz cover in an atmosphere of nitrogen at a distance of 40 cm. and for a period of 35 minutes. A Cooper Hewitt laboratory lamp was used as a source of ultra-violet light.

Method of Testing Antirachitic Potency.

Young rats weaned at the age of 22 days were kept on the stock diet 2 to 5 days until they weighed from 45 to 50 gm. They were then changed to the McCollum¹ Diet 3143 for 14 days and kept in the dark room. On the 15th day we began to administer the preparation which we wished to test for curative properties.

A known amount of the test material was dissolved in ether and incorporated with a known amount of the rachitic diet. After allowing the ether to evaporate weighed portions of this food which would give the desired daily dosage of the test material were placed in small glass dishes and moistened with water. Each rat was transferred separately to a small cage and given the freshly moistened food. If the concentration of test material incorporated with the food was adjusted so that no more than 0.5 gm. of food was given daily, no difficulty was encountered in training the rats to eat the entire portion of food within 10 to 15 minutes. The doses were administered daily except Sunday. In the early experiments four rats were used for each test. One was killed at the end of 10 days, one at 15 days, one at 20 days, and one at 25 days. The line test of McCollum was made in the usual way. It was found that quantitative differences in antirachitic potency could be detected best at 10 days.

In later assays each material tested was given in graded dosages beginning with one which produced advanced cure and diminishing to one which produced little or no cure. Two animals were used for each dosage. They were killed at the end of 10 days. A degree of healing equal to ++ at the end of 10 days was accepted as the end-point. For most of the samples the preliminary test was

¹ See McCollum, E. V., Simmonds, N., Shipley, P. G., and Park, E. A., *J. Biol. Chem.*, **47**, 507 (1921).

confirmed by repeating the 10 day test on three dosages which produced healing of ++, better than ++, and little or no healing.

Relative Potency of Fractions.

Previous work with cholesterol of the degree of purity of Cholesterol A had indicated that a minimum daily dose of 2.5 mg. was necessary for ++ healing in 10 days. Therefore, in these first tests a daily dose of 2.5 mg. was used for all of the fractions examined. Of the eight materials, Fraction 1c, the acetone extract from the soaps, showed very little antirachitic activity, Cholesterol A gave a line test of about ++ in 10 days, Cholesterol B a positive test between ++ and +++, and Fraction 1a₁ a positive test of +++. The other samples, Fraction II, Cholesterol A sublimate, Cholesterol A residue, and the original Cholesterol W all gave results distinctly better than +++. In fact, by this test the bones from animals receiving daily 2.5 mg. of these materials showed at the end of 10 days very little variation from the normal. Obviously the heating of the more purified cholesterol in our attempt to purify further by sublimation had increased the activatability of both the sublimate and the residue.

Since Cholesterol A was our most highly purified material we used that as a standard with which to compare all our other samples. Three of these highly activatable samples, Cholesterol W, Cholesterol A sublimate, and Cholesterol A residue, were given in decreasing doses of 2 mg., 1.5 mg., 1 mg., and 0.5 mg. Again in the case of these three products the 0.5 mg. dose produced more advanced healing in 10 days than 2.5 mg. of Preparation A. When the dosages were diminished still further to 0.3 mg. and 0.1 mg., it was found that for these three substances a daily administration of 0.1 mg. produced a degree of healing which was comparable to that produced by 2.5 mg. of Cholesterol A.

Effect of Heating Cholesterol under Different Conditions.

The increased activatability of Cholesterol A after heating called for further investigation. We first attempted heating cholesterol in sealed tubes in an atmosphere of N₂ holding at different temperatures for different periods of time. 0.5 gm. portions of Cholesterol A were placed in heavy-walled tubes of

about 1 cm. bore. After passing N_2 through for 1 hour, these tubes were sealed and heated in an oil bath at 200° and 225° , and in a metal bath at 260° . The following treatments were carried out:

200°	for	2	hours,	for	25	hours.
225°	"	11	"	"	72	"
260°	"	15	"	"	55	"

We hoped by the long periods of heating at high temperatures to obtain even greater increases in activatability than before so we tested these materials in daily doses of 0.5 mg. and 0.1 mg. Contrary to expectations the samples heated in closed tubes, although they showed distinct increases in activatability, were none of them as potent as that which had been heated at 7 to 10 mm. pressure. By heating under this increased pressure the activatability was increased from 2 to 5 times but in no sample was there as great an increase in activatability as in the previous experiments when the cholesterol was heated under reduced pressure.

Effect of Pressure on Increased Activatability.

These results suggested that the failure to obtain the same degree of increased activatability in the sealed tubes was due to the higher pressure under which the cholesterol was heated. By means of a Cenco Hyvac pump and a Cenco vacuum control apparatus, it was possible to maintain constant pressures of N_2 at any point between 2 and 100 mm. We heated 0.5 gm. portions of cholesterol for 6 hours at 210 – 215° under pressures of 2 mm., 10 mm., 50 mm., and 90 mm. of N_2 . In each case the tube containing the cholesterol was placed in the *cold* oil bath and heated gradually to 210° after which it was held at 210 – 215° for 6 hours. The tubes were then raised from the bath and allowed to cool rapidly.

At the very low pressures, 2 mm., the cholesterol sublimed rapidly at 185 – 200° , so that one-half to three-fourths of the total amount was caught as sublimate on the water-cooled condenser. A few crystals were found in the outlet tubes. Only a small portion remained in the tube to be subjected to the temperature of the bath. This residue remaining in the tube was a creamy,

semicrystalline mass. When tested biologically it required 0.8 to 1 mg. of the sublimate and 0.3 mg. of the residue to produce a degree of healing equivalent to 2.5 mg. of the original purified cholesterol. This was repeated on another sample of purified cholesterol with essentially the same results.

On the other hand, at 10 mm., 50 mm., and 90 mm. pressure of N_2 the amount of sublimate collected was very small and it accumulated slowly. When tested biologically both the residue and sublimate produced healing of ++ with daily dosages of 0.1 to 0.2 mg.

In later experiments purified cholesterol heated in an atmosphere of N_2 at atmospheric pressure acquired a potency of the same order as these samples heated at lower pressures of N_2 . The pressure at which the cholesterol is heated therefore appears to be unimportant, at least when the pressure does not exceed atmospheric pressure and when a current of gas is swept over the molten cholesterol. It apparently is necessary, however, that the cholesterol shall be subjected to a temperature above the melting point for an appreciable time since at the very low pressures of 2 mm. the cholesterol sublimed almost as it melted and in that case the sublimate was very little different in activatability from the original cholesterol.

This observation was confirmed when we again heated the cholesterol at 50 mm. pressure but the tube containing cholesterol was not immersed until the oil bath had reached 210° . Then again the cholesterol sublimed almost completely as it melted. And in that case again the sublimate was very little superior in activatability to the original cholesterol. Further confirmation of these biological tests was found in the spectrographic studies described in the next paper of this *Journal*.

Effect of Time of Heating.

Since pressure appeared not to be significant, we heated portions of cholesterol in N_2 at atmospheric pressure for varying periods of time to determine whether the change responsible for the increased potency occurs rapidly after the cholesterol melts or whether it is a slow reaction. We placed two tubes containing samples of purified cholesterol in a cold oil bath and passed N_2 through the system for 30 minutes before beginning to heat. At

165° one tube was removed; the other was held at 165° for 2 hours. When tested biologically the sample which had been simply melted showed double potency while that which had been heated 2 hours was equivalent to some of our samples heated under reduced pressure requiring a dose of 0.1 mg. daily to produce a ++ healing in 10 days. From these results as well as the previous ones it appears that the time factor is important in the change from the inactivatable to the activatable form. Some increase in provitamin D potency has always been observed in cholesterol heated to the melting point, but the maximum activatability is attained after 1 to 2 hours. Continued heating produces little increase or decrease in activatability if oxidative changes are controlled. A sample of Cholesterol A which had been heated at 7 to 10 mm. of N_2 for 15 hours at 220–225° showed only a slightly greater increase in activatability, in this case a minimum dose of 0.05 mg. daily being required.

It is probably significant that in our heated samples of cholesterol a minimum curative dose of 0.05 to 0.1 mg. is the maximum potency obtained under whatever conditions the heating was done. We frequently obtained less potent preparations, but never more potent. Our repeatedly consistent results with one and the same preparation irradiated at different times ruled out the probability that the lower potency of some samples was the result of variations in activation during exposure to ultra-violet light. Neither had we been able to demonstrate that a specific pressure or temperature was the deciding factor. After a 2 hour period the time of heating seemed unimportant. We had suspected, however, that our most potent preparations were obtained when a trace of air was admitted to the system. In preparing our heated samples we had tried to avoid oxidation because we had early observed that cholesterol heated in an open dish was completely changed to a brown transparent mass with a distinct caramel odor. Such a product was not activatable. This did not rule out the possibility that a trace of oxygen might be involved in the change of inactivatable cholesterol to the activatable form. In preparing our next samples we heated one portion taking every precaution to remove oxygen. A new system of wash bottles was set up. The N_2 was permitted to bubble rapidly through the system for 1 hour when the escaping gas was tested

by passing it through an alkaline solution containing freshly precipitated manganous hydroxide. If after bubbling the N_2 coming from the outlet tube through this alkaline mixture for 5 minutes no darkening of the precipitate occurred, the gas was considered O_2 -free. The outlet from the tube containing cholesterol was then sealed with a water trap and the oil bath was heated to 185° and held at 185 – 190° , for 2 hours.

In the case of the second sample, the manganous hydroxide test was applied after N_2 had been passed through the system for 15 minutes. A slight browning of the precipitate developed. At this stage the oil bath was heated to 185° and held for 2 hours.

When tested biologically the minimum curative dose of the first preparation was 0.4 mg. and of the second 0.1 mg. These results apparently confirmed our suspicion that a trace of oxygen aids in producing the increased activatability.

We next investigated the effect of heating cholesterol in a tube, stoppered with a cotton plug. Portions of Cholesterol A were thus heated at 190° for periods of 1 hour, 2 hours, and 6 hours. After 1 hour's heating the cholesterol was creamy yellow, but distinctly crystalline. After 2 hours heating there was more discoloration and the cholesterol on cooling presented a semicrystalline appearance much like the residue of our potent samples heated for the longer periods under reduced pressure. The 6 hours heated sample, on the other hand, cooled to a transparent brown solid entirely lacking any crystalline structure. But even this last sample when irradiated and tested was potent in a 1.5 mg. dose, thus showing some degree of increased activatability. The 1 hour heated preparation was potent in a 0.05 mg. daily dose. The 2 hour sample produced a ++ healing with a 0.3 mg. dose. It appears then that heating in the open tube brings about increased activatability but parallel with this process is a destructive action, probably oxidation, which again reduces the antirachitic potency. This destructive action of heat increases rapidly with an increase in temperature. A portion of cholesterol heated at 250 – 270° for 3 hours underwent greater changes than did that heated at 185 – 190° for 6 hours.

Such an increase in antirachitic activatability of a purified cholesterol is certainly suggestive that the provitamin D potency resides in the cholesterol molecule itself, unless we grant that

ergosterol is formed from cholesterol. The fact that ergosterol heated under exactly the same conditions is reduced in activatability makes this latter supposition quite untenable. Also the spectra of our heated cholesterol preparations show no ergosterol bands, but general absorption only.

It remained for us to prove that cholesterol purified by other means than boiling with base and recrystallization, especially those methods which are said to destroy ergosterol, still retained some antirachitic potency and also that cholesterol so purified and then heated under the conditions described acquired the same degree of antirachitic activatability as did our cholesterol purified by less rigorous treatment.

Purification by Bromine Treatment.

50 gm. of the commercial cholesterol were purified by bromine treatment employing the method described by Bills, Honeywell, and MacNair (1). Both the dibromide and the cholesterol recovered by reduction of the dibromide were tested biologically in 5, 10, and 15 mg. daily dosages. The irradiated cholesterol produced no healing in the 5 mg. dose, but in the 10 mg. dose brought about a degree of healing better than our usual end-point of ++. The dibromide produced no trace of healing in any dosage administered.

Purification with Potassium Permanganate.

We also treated the commercial cholesterol with KMnO_4 in acetone in the manner described by Bills, Honeywell, and MacNair (1). 100 gm. of Cholesterol W were dissolved in 1200 cc. of hot acetone and after the addition of 1 gm. of finely powdered potassium permanganate the solution was boiled under a reflux for 1 hour. The hot solution was then filtered to remove the manganese dioxide and again 1 gm. of potassium permanganate was added. This procedure was repeated a third time after which the recovered crystals were further purified by three more recrystallizations from acetone. We irradiated and fed the original Cholesterol W and also that same cholesterol treated once, twice, and three times with potassium permanganate. The untreated cholesterol produced a cure of ++ with 0.1 mg. dose, the once treated cholesterol with 1.5 mg., and the twice and three times

treated with a 3.0 mg. dose. The first treatment reduced the activatability to one-fifteenth that of the original cholesterol, the second treatment again reduced it by one-half, but the third treatment apparently produced no further change.

Purification by Acetylation.

100 gm. of the commercial product were boiled under a reflux condenser $1\frac{1}{2}$ hours with 300 cc. of acetic anhydride. After cooling, the crystals were filtered off and recrystallized from acetone three times. Then 30 gm. of the acetate were hydrolyzed by boiling 1 hour in 300 cc. of 5 per cent H_2SO_4 in alcohol. The cholesterol recovered from the cooled solution was recrystallized four times from alcohol. Another 30 gm. were hydrolyzed by boiling in 400 cc. of 5 per cent NaOH. The recovered crystals in this case were recrystallized four times from alcohol.

These three products, the acetate, the acid hydrolysate, and the alkaline hydrolysate were tested for antirachitic potency after irradiation in the usual manner. It required 2 mg. of the acetate (equivalent to 1.75 mg. of cholesterol), 4 mg. of the acid hydrolysate, and 1.5 mg. of the alkaline hydrolysate to produce a +++ healing. This result confirmed an earlier experiment in which we found the alkaline hydrolysate slightly more potent than either the acetate or the acid hydrolysate of the acetate.

This effect of alkaline treatment in enhancing the antirachitic activatability of purified cholesterol was again confirmed by boiling for 5 hours a sample of the potassium permanganate-treated cholesterol in a 5 per cent solution of sodium hydroxide in alcohol. After recovering the cholesterol it was recrystallized three times from acetone and was still twice as active when irradiated as the original purified cholesterol. The cholesterol recovered from the filtrates showed four times the activatability of the original purified cholesterol.

By none of the methods of purification employed were we able to produce a cholesterol which had *no* provitamin D activity. By increasing the dosages sufficiently we were able in every case to produce typical calcification of the bones—and this, in spite of the fact that spectral analysis of these products showed no trace of ergosterol bands. In this finding we confirm Bills, Honeywell, and MacNair (1) who also reported that after a second treatment

with bromine or with potassium permanganate the recovered cholesterol when irradiated was capable of curing rickets when the dosage was increased 30 times above the minimum curative dose of the original cholesterol. We obtained a minimum curative dose by our method of assay with the KMnO_4 -treated cholesterol of 30 times that of Product A, but with the bromine-treated cholesterol it was necessary to increase the dosage by 70 times. We subjected our cholesterol to potassium permanganate treatment only three times while Kon, Daniels, and Steenbock (2) repeated the procedure five times. Steenbock reported that his product was completely inactivatable while our product produced a ++ cure with a 3 mg. daily dose. Since the biological assay showed no further reduction in provitamin D after the second treatment with potassium permanganate, we assume that a fourth and fifth repetition of the procedure would have had little or no effect. We feel we are safe in assuming like Bills, Honeywell, and MacNair (1) that the observed activatability of such purified products must be due, in part at least, to something other than ergosterol although we grant that traces of ergosterol which cannot be detected even spectrographically may be present. The question arises why there is such a difference in the antirachitic potency of these several purified products if the activity resides in the cholesterol molecule *per se*. We have already demonstrated that heat changes cholesterol of low activatability to a more activatable form. It is not improbable that other procedures may establish an equilibrium between the less activatable and more activatable forms at different levels. It is also true that cholesterol as well as ergosterol is destroyed in the purification methods employed, but the former to a lesser extent. A comparison of the melting points and minimum curative doses of the various preparations shows no correlation whatever between melting point and antirachitic activatability.

Effect of Heat on These Specially Purified Cholesterols.

The bromine-purified cholesterol and our first purified Cholesterol A were heated at the same time in a stream of N_2 at 165° for 2 hours. When tested biologically they gave similar results; *i.e.*, a cure of ++ with a daily dosage of 0.1 mg. Likewise the KMnO_4 -treated cholesterol, when heated in the same way also became potent to this extent.

In these preparations, we have used methods which are destructive to ergosterol. Yet we have been able by heating to produce in these products a provitamin D activity which, we grant, does not equal that of ergosterol, but which is equal to the potency of the original commercial product. The original Cholesterol W does show in a 2 per cent solution all four of the ergosterol bands. The spectrum of the heated cholesterol, on the other hand, shows no bands but general absorption in the region beginning at 2950 A.units, the position of the first ergosterol band. This it seems to us is conclusive proof that provitamin D activity resides in the cholesterol itself and that in augmenting the provitamin potency we have produced it from the cholesterol itself and not regenerated ergosterol.

It probably is significant that by this heating process we have never been able to secure a greater potency than a minimum curative dose of 0.1 to 0.05 mg. We have obtained less potent preparations but never more potent. This suggests that at this stage equilibrium is established between the activatable and the inactivatable forms.

This reaction of purified cholesterol to heat suggested the possibility that ethers or other condensation products are formed and that such condensation products are the cause of the increased activatability. Further information concerning the nature of the change which occurs when the cholesterol is heated would be available if we could duplicate or augment this increased activatability by chemical means.

Action of Dehydrating Agents on Cholesterol.

It seemed logical to investigate the action of various dehydrating agents. 0.5 gm. portions of Cholesterol A were mixed in separate tubes with 0.1 gm. of P_2O_5 and with 0.4 gm. of soda-lime and the tubes sealed. Another portion was mixed with anhydrous $CuSO_4$ in a small flask closed with a $CaCl_2$ tube. These were all suspended in an oil bath at 210° in such a way that the behavior of the materials could be observed. Reaction with the P_2O_5 came immediately when the cholesterol melted. A purple then brown color developed. After 15 minutes heating this tube was removed from the bath. The soda-lime and anhydrous $CuSO_4$ mixtures were heated 30 minutes. In each case droplets of moisture con-

densed on the top of the tube or flask. During the last 10 minutes of heating a brown distillate from the CuSO_4 mixture condensed on the neck of the flask. After cooling, the mixtures were extracted quantitatively with anhydrous ether. The product from the P_2O_5 mixture had entirely lost its crystalline character. It was a yellow waxy mass similar to oxysterol prepared by treatment with benzoyl peroxide. Judging from the increase in weight a phosphoric acid ester of cholesterol or of oxidized cholesterol had been formed. A positive organic phosphate test was obtained with this material.

The products removed from soda-lime and anhydrous CuSO_4 were both creamy in color but distinctly crystalline.

These products were tested in doses of 1 mg., 0.5 mg., and 0.3 mg. The preparation from P_2O_5 and CuSO_4 produced no healing in the 1 mg. dose. That obtained from the soda-lime treatment, however, showed some increase in activatability, 1 mg. being equivalent in effect to 2.5 mg. of Cholesterol A. Since very potent preparations have been obtained by heating 1 hour without the addition of CuSO_4 , we feel safe in assuming that the CuSO_4 effect was either negligible or destructive.

Action of Yellow Phosphorus.

Another sample of Cholesterol A was suspended in H_2O with bits of yellow phosphorus wire. The water was siphoned off and the moist mixture of cholesterol and phosphorus were allowed to remain in contact for 2 hours, the flask being shaken frequently. The cholesterol was then dissolved in ether, the ether solution washed three times with water and finally evaporated. The cholesterol had developed a yellow tinge and retained a garlic-like odor, but when tested showed an activatability about equivalent to the original Cholesterol A. This experiment was suggested by the well known effect of minute doses of yellow phosphorus in promoting bone growth.

Heating with Metallic Salts or Oxides.

Sabatier (3, 4) and Ipatiew (5) have drawn attention to the catalytic effect of the oxides and some of the salts of metals such as aluminum, tungsten, and thorium in producing unsaturated bonds by removal of water. We therefore tried heating chole-

terol with AlCl_3 , Al_2O_3 , and MoO_3 in the proportion of 1 gm. of cholesterol to 10 mg. of the catalyst. This was done in an atmosphere of N_2 . Although the spectra of these samples of cholesterol, especially the one heated with AlCl_3 showed even greater general absorption than the cholesterol heated without these additions, the antirachitic potency of all these samples, those heated with and without the added substances, was the same.

All these attempts to further increase the provitamin D potency of highly purified cholesterol preparations have been fruitless since the reagents used have been either negligible or destructive in their effects.

Attempts to Discover the Chemical Reactions Involved in the Increased Activatability.

We have made numerous attempts to discover what type of chemical reaction occurs when the cholesterol is heated. If we could learn whether the augmented activatability is due to polymerization, or increase in the number of unsaturated bonds, or a shifting of the position of a double bond it might help to elucidate some of the questions concerning the chemical nature of provitamin D and what the action of light is in activating it.

Color Reactions.

The Shear and Kramer (6) aniline hydrochloride test which the authors have used to identify so called uviol or vitamin D was used on many of our preparations. We found that all samples both non-irradiated and irradiated which were discolored, indicating the presence of oxidation products of cholesterol, gave a strong positive test. Fresh solutions of "pure" ergosterol both non-irradiated and irradiated gave negative results. The intensity of the test had no correlation whatsoever with vitamin D potency.

Rosenheim (7) has recently published tests by means of which he claims to be able to distinguish pure cholesterol from allo-cholesterol, β -cholesterol, and cholesterylene and each of the above from ergosterol. In trying to apply these tests in our studies we have not been able to confirm Rosenheim's claims as to the value of the method in detecting traces of ergosterol in presence of large amounts of cholesterol of various grades of purity.

Iodine Number.

A micro iodine number method devised by us when applied in this study showed no changes in the iodine number of the various forms of cholesterol here investigated. More detailed information on the method and results will be published later.

Digitonide Formation.

By digitonin precipitation identical values were obtained with both the heated and the unheated cholesterol used in these studies.

Refractometric Studies.

A change in refractometer reading would indicate a change in molecular weight. 1 per cent solutions in alcohol of potassium permanganate-purified cholesterol and of the same cholesterol after heating 2 hours at 190° were read at 25°. The heated sample was shown by biological assay to be 30 times more activatable than the unheated sample. The difference between the pure solvent reading and that of the 1 per cent solutions was in the case of the original sample 4.48° and of the heated samples 4.50°. There is then no evidence that polymerization is involved in the change from the inactivatable form of cholesterol to the activatable.

Changes in Optical Rotation.

Burian (8) gives the optical rotation of cholesterol as $[\alpha]_D^{25} = -29.92$ (4.6 gm. per 100 cc. of solution in anhydrous ether). We obtained for the same concentration the value $[\alpha]_D^{25} = -29.56$. For convenience we substituted the green mercury light for sodium. We found that a sample of ergosterol changed after irradiation for 35 minutes at 40 cm. distance from $[\alpha]_{Hg}^{25} = -101.8$ to -70.9 . Our potassium permanganate-purified cholesterol gave the value $[\alpha]_{Hg}^{25} = -38.04$. Heating this same cholesterol under 1 to 2 mm. N_2 at 220–225° for 2 hours produced no change. Heating at 7 to 10 mm. pressure for 2 hours decreased the levorotation to $[\alpha]_{Hg}^{25} = -37.5$. Heating in the open tube decreased it still further to $[\alpha]_{Hg}^{25} = -35$. In these particular samples the increase in activatability was paralleled by a decrease in levorotation. Whether this change in optical rotation is related to the change from the inactivatable to the activatable form is a question. At any rate

one or more asymmetric carbon atoms in the cholesterol molecule appear to be sensitive to high temperatures, even though there are no profound oxidative changes.

DISCUSSION.

The experimental evidence presented above and as correlated in the next paper clearly shows that highly purified cholesterol is still activatable to antirachitic activity, but that it is made still more so when the solid cholesterol is molten under certain conditions of time, temperature, and oxygen exposure. We have not been able to find an amount of ergosterol in these preparations comparable to the provitamin activity. In fact, we have not been able to detect ergosterol in the highly purified product, nor in the more activatable forms obtained therefrom. Our conclusions are that the cholesterol is modified chemically in the melting and heating process and that it is not changed to ergosterol. Thus far we have not been able to throw light on the nature of the change involved. There are here possibilities of numerous tautomeric rearrangements as well as inter- and intramolecular oxidations and reductions. The studies by Diels and Abderhalden (9), Diels and Linn (10), and Diels, G6dke, and K6rding (11), suggest that we may be producing β -cholesterol and cholestanon. If cholestanon is formed it is equally likely that cholestan, dihydro-cholesterol, ψ -cholesterol, and allocholesterol may be formed in variable amounts. Windaus (12), however, in 1912 considered the cholesterol nucleus relatively stable at temperatures below 300°. In a later paper (13) he reports the conversion of cholesterol chloride into allocholesterol by boiling the same with potassium acetate under anhydrous conditions in absolute alcohol. He also reports converting cholesterol in part into γ -cholesterol by boiling for 8 hours with sodium alcoholate in absolute alcohol. By heating dry cholesterol with freshly reduced nickel at 220° for 8 hours he obtained cholestanon. Heilbron, Morton, and Sexton (14) and Heilbron and Sexton (15) report the formation of water, hydrogen, hydrocarbons, cholestenone, and ψ -cholestene in the dry distillation of cholesterol in a current of carbon dioxide. It is easily possible that one or more of these products are formed in small amounts in the treatment employed by us and that the increased activatability is due to one or more of these. We are

continuing these investigations on the chemical and physical as well as the biological phases.

Our observations are at least in part confirmed by the earlier observations as published by Bills, Honeywell, and MacNair (1). It may be recalled that they also found ergosterol in the commercial cholesterol, but that the latter could not be obtained in an inactivatable form by the bromine treatment. They also concluded that their purified product must contain some other provitamin D than ergosterol.

SUMMARY.

A commercial cholesterol obtained from spinal cord was found to contain ergosterol. This when purified by four different methods always yielded a cholesterol which appears to be free from ergosterol, but which still possesses a provitamin D activity of one-seventieth to one-twenty-fifth that of the commercial product used.

When further attempts were made to purify the purified products by sublimation under diminished pressure in nitrogen both the sublimate as well as the non-sublimable residue possessed about 25 times the provitamin D activity of the purified material used.

Numerous repetitions of the heat treatment under various conditions of temperature, time, and pressure lead to the conclusion that the best conditions for increasing the activatability by the heat treatment are heating with little or no oxygen present at temperatures slightly above the melting point for periods of 1 to 3 hours.

In parallel with the changes in antirachitic activatability we have observed changes in melting point, absorption spectra, and slight changes in optical rotation. We have not been able to detect any chemical change in the heated products by color reactions, iodine number estimations, or refractive index readings.

We conclude that provitamin D activity is not limited to ergosterol, but that it may be a general property in varying degrees of various sterols or certain forms of those sterols.

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ABSORPTION SPECTRA STUDIES ON CHOLESTEROL AND ERGOSTEROL.*

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PLATES 1 TO 4.

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In the previous paper it was shown that highly purified spinal cord cholesterol is still activatable to antirachitic activity when irradiated in the solid form by a Cooper Hewitt mercury vapor quartz lamp. Although the activatability is decreased by the purification methods employed, nevertheless these purified products can again be rendered at least 25 times more activatable if heated under the proper conditions slightly above the melting point of cholesterol. Inasmuch as provitamin D activity has been claimed to be due specifically to ergosterol (1-4) our results might be interpreted as due to the presence of or formation of ergosterol in our preparations and as it is well known that the absorption spectrum of ergosterol is a very characteristic one we considered it desirable to conduct absorption spectra studies on our products in order to obtain physical evidence for or against this interpretation.

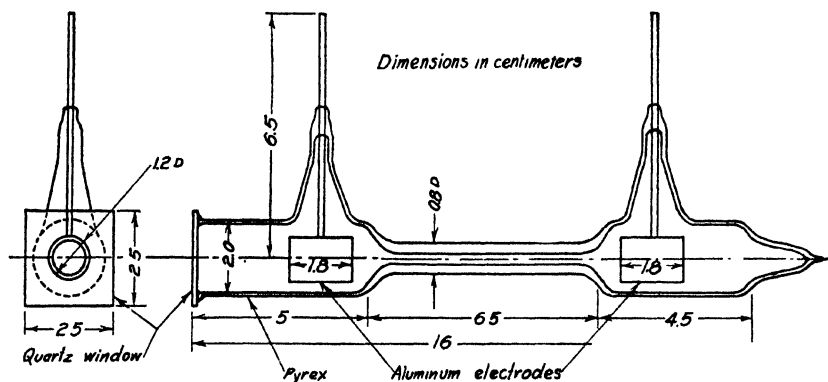
As a source of light the continuous radiation from hydrogen in the violet and ultra-violet region was utilized (5-7). The gas was contained in a Geissler tube of simple design (Text-fig. 1) so arranged that the luminous thread of the capillary portion was projected end on through a quartz window upon the slit of the spectrograph. The source tube contained pure electrolytic hydrogen at a pressure of about 15 mm. and was rendered luminous by excitation from the secondary of an open core transformer, the primary of which through a current-limiting rheostat was con-

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nected to 110 volt a.c. mains. Between the source and the spectrograph was a simple quartz condensing lens and adjacent to the slit the absorption cell (Baly tube) containing the solutions being tested.

The spectrograph was one by Adam Hilger, Type E3, recording the spectrum from 2100 to 7000 Å. units well within the extent of a 10 inch plate. Wratten panchromatic plates were used, developed for 4 minutes with contrast glycine developer containing bromide.

The materials examined were dissolved in ether and placed in the Baly tube having quartz windows. The depth of liquid



TEXT-FIG. 1. Hydrogen-filled tube for continuous ultra-violet ray spectrum. One-half actual size.

through which the light passed could be varied at will by adjusting the position of the plunger. Unless otherwise specified 2 minute exposures through a column of liquid 20 mm. in depth were employed.

The preparations studied in the previous paper and in this were the following: (1) A commercially pure cholesterol from the Wilson Laboratories (Cholesterol W); (2) this when purified by boiling with alkali and extraction with ether gave two fractions, one the main final recrystallized product (Cholesterol A) and another in the emulsion layer (cholesterol Fraction II); (3) "cholesterol Br-treated" was obtained by purifying Cholesterol W by means of bromine treatment; (4) cholesterol KMnO_4 -treated"

was obtained from "Cholesterol W by destruction of ergosterol by KMnO_4 . For more detailed information see the previous paper (8).

In Fig. 1 we made a preliminary comparison of the spectra of our cholesterol preparations with those of ergosterol. As we expected, the purified cholesterol was more transparent to ultra-violet than the original commercial product, Cholesterol W. The very striking change in the absorption of the purified cholesterol when it had been heated and also the great similarity in the spectra of the heated cholesterol and of Fraction II, both highly activatable preparations, to that of ergosterol, at least offered some confirmation of our biological reactions, described in the previous paper. In making these exposures the slit of the spectro-scope was very narrow and the concentrations were relatively high, 5 per cent cholesterol and 2 per cent ergosterol. We next used a slightly wider slit and varied the concentrations of the various preparations, diminishing each to the point where no absorption different from that of the pure solvent occurred.

Fig. 2, in which diminishing concentrations of Cholesterol W were examined is self-explanatory. In dilutions of 3, 2, and 1 per cent, the typical ergosterol bands at approximately 2930, 2800, 2720, and 2600 \AA .units became evident. From this picture we might assume, like Rosenheim and Webster, that the pro-vitamin D potency of Cholesterol W resided entirely in the ergosterol present as a contaminant.

Further investigation, however, leads us to believe that there are at least two substances in Cholesterol W which influence the picture, one ergosterol and the other a modified form of cholesterol which produces general absorption and that both of these substances impart to Cholesterol W its antirachitic activatability. When, for example, we compare the spectra of Cholesterol W with those of ergosterol (Figs. 2 and 3) it appears that a 2 per cent solution of Cholesterol W was equivalent to a 0.002 per cent solution of ergosterol except for a difference in end absorption and that in antirachitic activatability this ergosterol should have been 1000 times as potent as Cholesterol W. This particular lot of ergosterol, however, when tested biologically proved to be only 100 times as activatable as the cholesterol. The end absorption in the case of Cholesterol W then appears to be of fundamental significance.

Again we always observed that the four bands were never as clearly defined in the spectra of the three samples of Cholesterol W examined as in the case of ergosterol (compare Figs 2 and 3). It appears as though in Cholesterol W a general absorption in this region obscured the bands. We mixed cholesterol of low antirachitic activatability, which had been purified by bromine treatment and which showed no ergosterol bands, with ergosterol in the proportion of 1000:1. This mixture should have the same degree of antirachitic potency when irradiated as Cholesterol W. Yet when compared with the spectrum of Cholesterol W in the same concentrations the picture is quite different (Fig. 4). In the higher concentrations of Cholesterol A + ergosterol (5 to 3 per cent Cholesterol A + 0.005 to 0.003 per cent ergosterol) there is absorption between 2600 and 2800 Å.units due to a merging of three of the bands, while beyond 2600 the light comes through. The band at 2930 appears distinctly, but the other three are only faintly suggested. At the concentration of 2 per cent Cholesterol A + 0.002 per cent ergosterol, the four bands become clear. This picture is comparable to pure ergosterol in the same concentrations.

In the 5 to 3 per cent solutions of Cholesterol W on the other hand, there is complete absorption beginning sharply at 2980 Å.units. In the 2 per cent solution the first ergosterol band at 2930 appears and in the 1 per cent solution all four bands become apparent. This is what we might expect if in Cholesterol W there are two substances present—one causing general absorption, and the other ergosterol—and it is only after the substance causing *general* absorption is sufficiently diluted that the weaker ergosterol bands can be seen.

The substance in Cholesterol W, which causes general absorption and which has antirachitic potency when irradiated, was concentrated, we believe, in our Fraction II. This fraction separated in an emulsion layer at the second stage of purification of our original Cholesterol W. After the cholesterol had been boiled in a solution of sodium hydroxide in alcohol, the recovered crystals were dissolved in ether and the ether solution was washed several times with water. With every washing an emulsion layer formed which it was impossible to break either by adding ether or by long standing. The cholesterol in these emulsion layers was recovered as Fraction II.

When irradiated and fed to rats on a rachitic diet Fraction II had about the same antirachitic potency as the original Cholesterol W and yet it showed no ergosterol bands (Fig. 5). There was only general absorption beginning at about 3050 Å. units and diminishing with decreasing concentrations until a 0.1 per cent solution gave essentially the same picture as the pure solvent.

This finding that Fraction II, although of the same order of provitamin potency as Cholesterol W, showed no ergosterol bands was given added importance when we found that our heated samples of Cholesterol A behaved in precisely the same manner (Fig. 6). Instead of bands there was general absorption beginning at this particular concentration (3 per cent) at 3250 Å. units and gradually fading out with diminishing concentrations. We have some evidence of a similar change in cholesterol when it is heated with alkali.

This change in cholesterol which caused general absorption was always correlated with the increase in antirachitic activatability. On the other hand those samples of heated cholesterol which when irradiated did not show increased curative effect likewise showed little or no change in their spectra. For example, we introduced portions of Cholesterol A into tubes and passed nitrogen through the tubes for 1 hour. The tubes were then sealed and heated for periods of time varying from 2 hours to 72 hours and at different temperatures varying from 200° to 260°. These specimens showed only slight increases in activatability (8). When examined spectrographically they showed very little change from the original Cholesterol A.

Likewise when Cholesterol A was heated under very low pressures of 1 to 2 mm., in which case most of the cholesterol sublimed within 15 minutes, the sublimate was not changed from the original Cholesterol A either in activatability or in its spectrographic picture. Whenever we heated cholesterol alone and obtained increased activatability there was always a marked general absorption in the ultra-violet beginning at 3100 to 3300 Å. units. If no such increase in activatability occurred we obtained no general absorption. On the other hand, if we heated cholesterol with reagents such as P_2O_5 and $CuSO_4$ which destroyed considerable cholesterol, then we frequently had strong general absorption with no increased activatability. These facts are illustrated in Fig. 7.

Here the strong similarity between Fraction II and an activatable heated preparation is shown in D, E, F, and G. Cholesterol A heated in the sealed tube, on the other hand, shows very little difference from the original Cholesterol A. See J and K.

When CuSO_4 and soda-lime were added the cholesterol underwent more profound changes, and strong general absorption was observed. But these preparations were of *low* potency when irradiated. Probably this is the type of oxidation products described by Kon, Daniels, and Steenbock (4) as causing general absorption.

Schlutz and Ziegler (9) in 1926 obviously observed the same change in the spectrum of cholesterol when it was heated. They found that: "Apparently these absorption bands are characteristic of the hydrated cholesterol while the anhydrous form shows no bands but general absorption." Treating the anhydrous form with water and evaporating to dryness on the water bath yielded the hydrated form again. Undoubtedly the bands described by them are ergosterol bands. We have not found such a reversion of the heated material to take place readily. A heated sample of cholesterol was dissolved in hot 95 per cent alcohol and slowly evaporated to dryness. This when irradiated and tested biologically was not reduced in antirachitic potency and also still showed the same degree of general absorption.

Since our first purified Cholesterol A was not subjected to rigorous treatment it seemed important to employ some of the methods described by other workers as completely destroying all antirachitic activatability such as bromination or boiling with KMnO_4 in acetone. Kon, Daniels, and Steenbock (4) have claimed that cholesterol treated three times with KMnO_4 has no ergosterol and cannot be rendered antirachitic. Rosenheim (3), Windaus (1), and Pohl (2) agree that cholesterol which has been treated with bromine likewise loses all activatability. We purified one lot of Cholesterol W by bromine treatment as described by Bills and another by a triple treatment with KMnO_4 in boiling acetone. When irradiated both of these samples produced calcification in dosages 70 to 30 times that required of the original Cholesterol W. These results confirm those reported by Bills, Honeywell, and MacNair (10). We do not assert that this low activatability may not be due to a trace of ergosterol, although we do not believe

that this is the fact since we have other evidence of highly potent cholesterol preparations which show *no* bands.

Again, we heated both of these purified preparations in an atmosphere of N_2 at 185° and increased the activatability so that a 0.1 mg. dose was adequate for a cure of ++ when administered for 10 days after a 14 day preliminary period on the rachitic diet. Fig. 8 shows the spectra of Cholesterol W, of the dibromide, of the reduced dibromide cholesterol, and of the heated reduced dibromide cholesterol. Tested biologically, Cholesterol W produced a cure of ++ in 0.1 mg. daily dose; the dibromide was entirely inactive in 15 mg. daily dosages; the reduced dibromide was potent in 7 mg. daily dosage; the heated reduced product was again potent in 0.1 mg. dosage. The same increased activatability was demonstrated in the case of the $KMnO_4$ -treated cholesterol after heating. In fact these results have been repeated so many times by two different workers that we can have no doubt of their accuracy.

SUMMARY.

The commercial preparation of spinal cord cholesterol studied shows the four typical absorption bands of ergosterol. This product appears to possess provitamin activity due to the presence of ergosterol and of another substance or substances, probably in part cholesterol itself and in part a modified cholesterol of unknown chemical character.

Purification of the commercial cholesterol by repeated washings and recrystallizations, by bromine treatment, or by boiling with potassium permanganate in each case yields a product which has no absorption bands corresponding to ergosterol and which shows no general absorption in the ultra-violet region. These products are nevertheless activatable although only one-seventieth to one-thirtieth as potent as the original commercial cholesterol.

When these purified products are heated slightly above the melting point under conditions that avoid appreciable oxidation every one of the purified products changes to practically 25 to 70 times the activatability of the presumably purer cholesterol. The minimum curative dose of all heated cholesterol preparations by whatever means originally purified was 0.05 to 0.1 mg. Parallel to the increases in activatability a strong general absorption is

observed in the ultra-violet region. In the absorption spectra of these more activatable forms there are however no bands whatever and hence it is not probable that the increased activatability is due to ergosterol.

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EXPLANATION OF PLATES.

PLATE 1.

FIG. 1. Unless otherwise specified the depth of solution is 20 mm. and the time of exposure 2 minutes. A, empty tube; B, solvent; C, 5 per cent Wilson cholesterol; D, 5 per cent Cholesterol B obtained therefrom; E, 5 per cent Cholesterol A obtained from Wilson product; F, 5 per cent of unsublimed residue from 15 hours heating at 225° of Cholesterol A; G, 5 per cent of Fraction II cholesterol obtained in purifying the Wilson product; H, 5 per cent of same but exposed for 4 minutes; I, 2 per cent ergosterol, 20 mm. for 4 minutes; J, same, but for 2 minutes; K, same, 10 mm. for 2 minutes; L, same, 5 mm. for 2 minutes.

FIG. 2. 5, 4, 3, 2, 1, and 0.5 per cent concentrations in anhydrous ether of our second lot from the Wilson Laboratories.

PLATE 2.

FIG. 3. A, empty Baly tube; B, anhydrous ether; C, D, E, F, G, H, I, and J are 0.01, 0.008, 0.005, 0.004, 0.003, 0.002, 0.001 and 0.0005 per cent concentrations of ergosterol in anhydrous ether.

FIG. 4. A, solvent; B, 5 per cent cholesterol from dibromide + 0.005 per cent ergosterol; C, same, but 3 per cent + 0.003 per cent; D, same but 2 per cent + 0.002 per cent; E, same but 1 per cent + 0.001 per cent; F, 5 per cent Wilson cholesterol; G, 3 per cent same; H, 2 per cent same; I, 1 per cent same.

PLATE 3.

FIG. 5. A, 0.004 per cent ergosterol in anhydrous ether; B, same of ergosterol previously irradiated in the solid form; C, D, E, F, and G are 2, 1, 0.5, 0.25, and 0.12 per cent concentrations of our "Fraction II" cholesterol obtained in purifying the Wilson product; H, anhydrous ether alone. The slight irregularities observed in this plate are due to using alternately two different Baly tubes.

FIG. 6. A, B, C, D, E, and F are 3, 2, 1, 0.5, 0.3, and 0.1 per cent concentrations of our heated purified product, Cholesterol A. For the method of preparation of the product and the subsequent treatment see the text. G, ether.

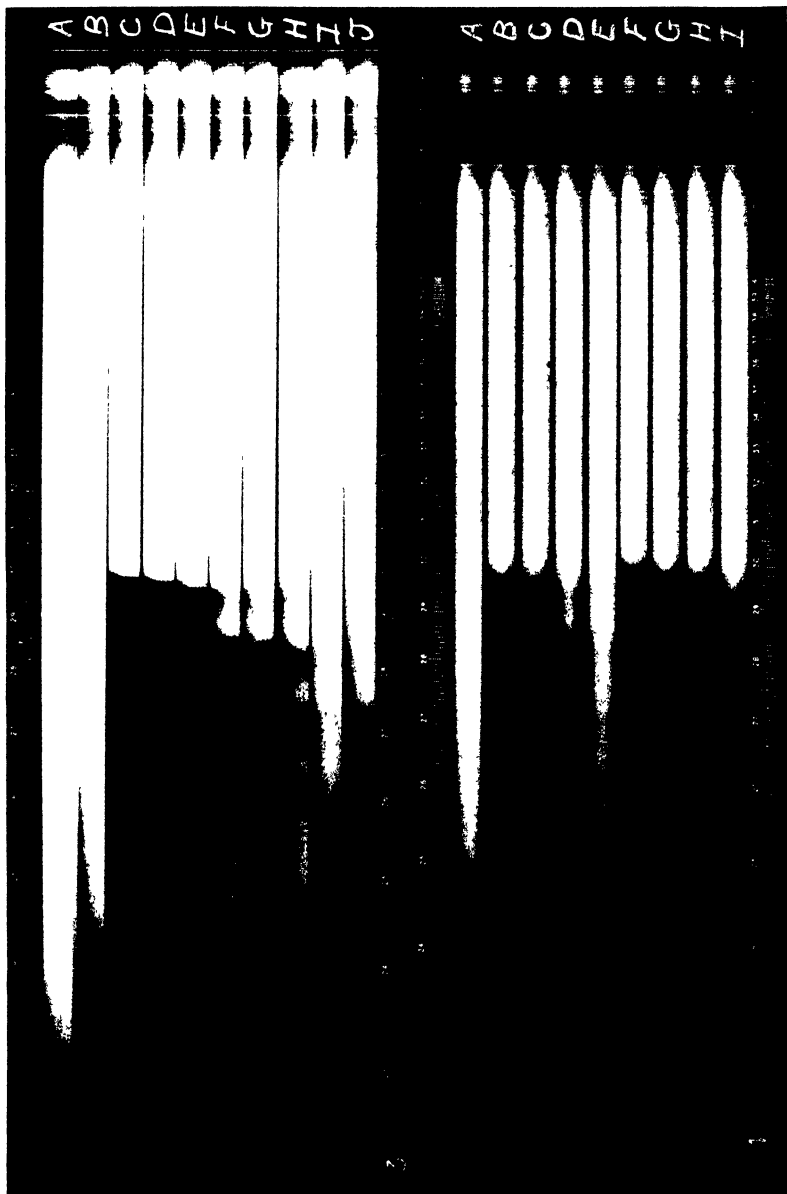
PLATE 4.

FIG. 7. A, 3 per cent irradiated Wilson cholesterol; B, 3 per cent Cholesterol A (non-irradiated); C, same irradiated; D, 3 per cent Fraction II cholesterol (non-irradiated); E, same irradiated; F, 3 per cent Cholesterol A, previously heated at 7 to 10 mm. pressure (non-irradiated); G, same irradiated; H, 3 per cent Cholesterol A heated with CuSO_4 ; I, 3 per cent Cholesterol A heated with soda-lime; J, 2 per cent Cholesterol A heated at 200° at high pressure for 25 hours (non-irradiated); K, same irradiated; L, solvent.

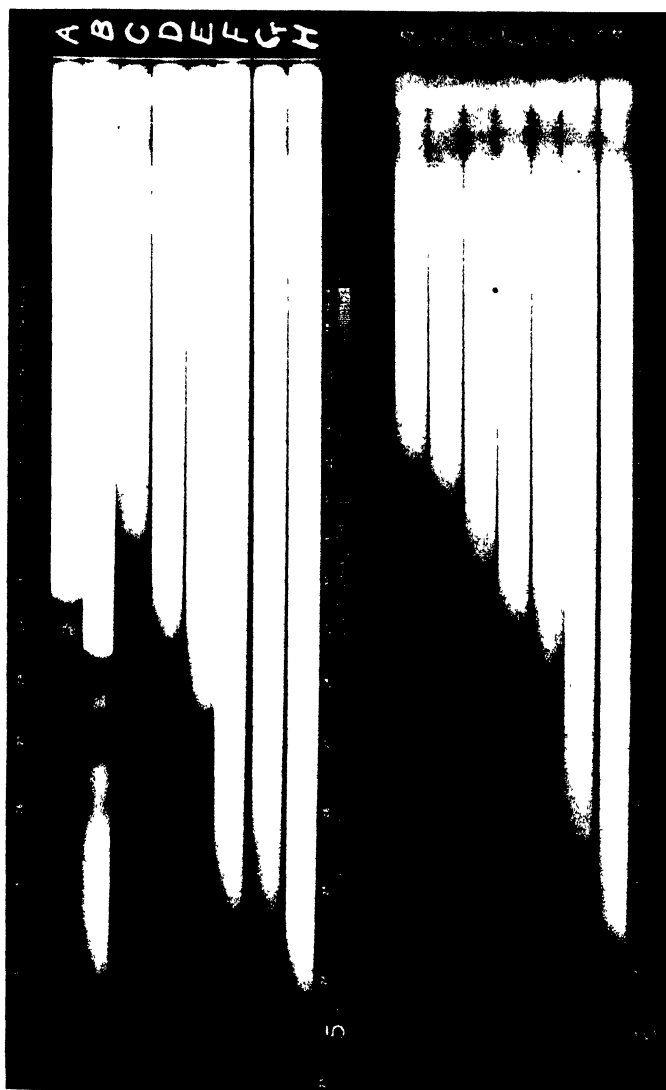
FIG. 8. Unless otherwise stated the depth of solution used is 20 mm. A, solvent; B, 6 per cent Wilson cholesterol; C, same, but 10 mm. depth; D, 3 per cent cholesterol dibromide; E, same, but 10 mm. depth; F, 6 per cent cholesterol from the dibromide; G, same, but 10 mm. depth; H, 3 per cent cholesterol from the dibromide, and then heated for 2 hours at 165° before dissolving in ether; I, same, but at 15 mm. depth; J, same, but at 10 mm. depth.



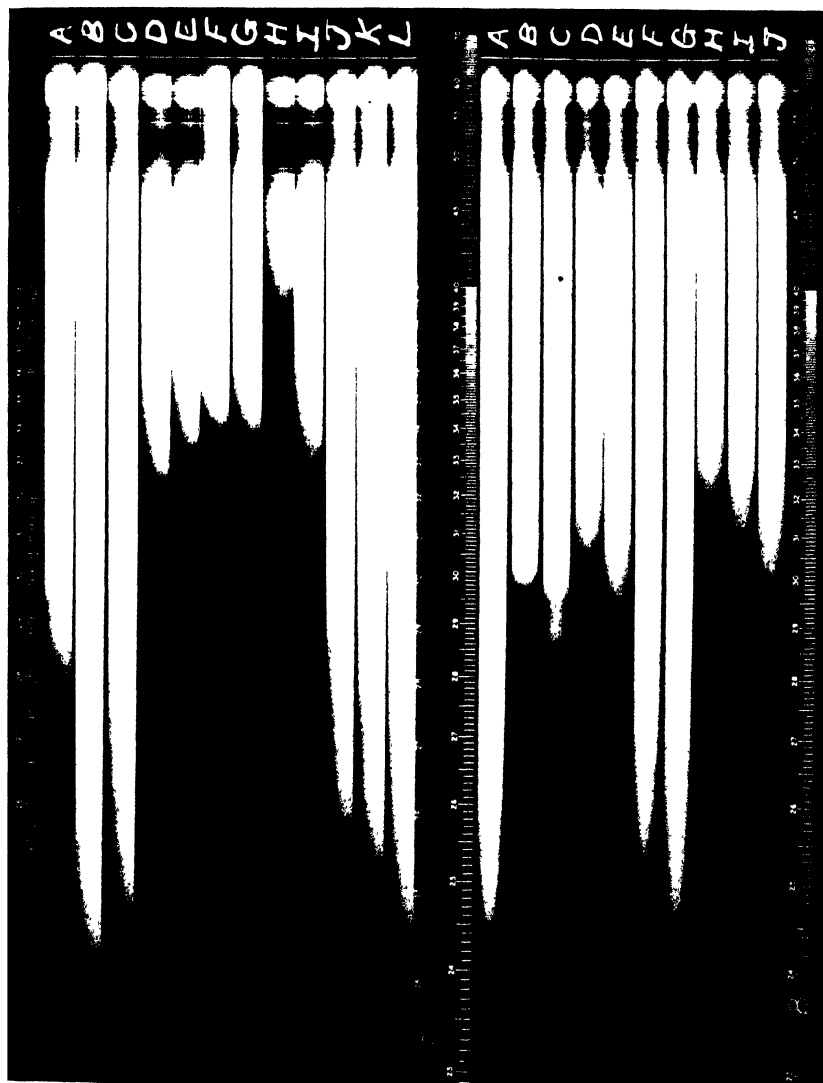
(Koch, Koch, and Lemon: Absorption spectra studies.)



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THE INFLUENCE OF PROTEIN METABOLISM ON THE CONVERSION OF CREATINE TO CREATININE.

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During the course of some studies on the physiology of the liver with reference to creatine-creatinine metabolism, I was surprised to find several dogs in which continued administration of creatine failed to give rise to an increase in the urinary excretion of creatinine. Since these observations did not correspond to the well established experiments of others, I sought the cause in the different details of the investigations. Two differences in our methods were apparent: creatine was administered intravenously instead of orally and the protein content of the creatinine-free diet was considerably lower than that used by other observers. Experiments designed to check these two points proved so interesting and conclusive that they seemed to warrant publication.

In the experiments of Benedict and Osterberg (1923) three dogs, having undergone a preliminary period of creatine-free feeding, were given daily for 5 to 10 weeks a dose of creatine of 0.45 to 0.65 gm. This period was followed by a period of observation of 3 to 7 weeks. The basal diet was held uniform from beginning to end, and throughout each experiment daily determinations were made of the output of nitrogen, creatine, and creatinine. For the 1st week of creatine feeding the small doses administered did not have any effect on the excretion of creatine or creatinine. The succeeding weeks showed an excretion of about 50 per cent of the creatine as such and the average daily excretion of creatinine entered, with the 2nd week, on a gradual rise. This rise continued as long as the creatine was given and at its height the daily elimination of creatinine was about 33 per cent more than that of the preliminary period. When the creatine was finally withheld, abrupt cessation of elimination of creatine was noted. The elimination of creatinine fell gradually and as late as 7 weeks after the last administration of creatine the creatinine of the urine definitely exceeded the original value. These results establish the origin of creatinine from creatine and

emphasize the exceptional slowness and perhaps the complicated processes involved in this reaction.

In view of the continued increased excretion of creatinine after withdrawal of creatine, Benedict and Osterberg assumed that these results could not be attributed to changes in creatine incident to its administration orally. Bacterial action or alteration of absorption of creatine in the intestine could not have been a predominant factor in producing the changes noted, since they continued several weeks after the administration of creatine was discontinued. The animals also gained weight and showed retention of nitrogen during the periods of ingestion of creatine, and Benedict and Osterberg were inclined to attribute this to some specific effect of creatine as a food. Gain in body weight had not been observed during the preliminary feeding of cracker meal, milk, and casein in fixed amounts. The nitrogen content of this diet was 6.65 gm. for the dog weighing 14.4 kilos, 5.45 gm. for the dog weighing 11.6 kilos, and 4.45 gm. for the dog weighing 9.1 kilos. The last animal failed to show any marked increase in body weight and it appears unlikely that changes in body weight alone are a controlling factor in the conversion of ingested creatine to creatinine. In calling attention to the processes which may be involved in the formation of creatinine from creatine, Benedict and Osterberg suggested that different conditions of diet or of general nutrition might greatly alter the changes noted in their experiment.

Chanutin (1926) obtained similar results in experiments on man. Rose, Ellis, and Helming (1928) in long continued feeding experiments on man showed that both male and female subjects could convert creatine to creatinine. Similar to the observations of Benedict and Osterberg, their observations showed that approximately a third of the creatine metabolized yielded creatinine.

Methods of Investigation.

Two well nourished adult male dogs, which had been on a standard balanced diet for several weeks, were selected for the experiment. The animals were maintained on a definite dietary regimen and daily collections of urine were made. After withdrawal of the urine by catheter the bladder was washed with 20 cc. of sodium chloride solution and the washing was repeated if it returned colored with urine. This was done at 10.30 a.m., and during the period of administration, creatine was fed or injected intravenously at this time. The animals were returned to the metabolism cages and fed the weighed diet at 11.00 a.m.; free access to water was allowed at all times.

The total nitrogen of the urine was determined by the Kjeldahl process, the creatinine and total creatine and creatinine were determined by the method of Folin (1922), with the use of purified

creatinine as a standard. The creatine hydrate used in the experiment was a purified commercial product which contained only a faint trace of creatinine and 1 gm. of the dried material yielded 751 mg. when determined as creatinine. For administration to animals this was dissolved in water with the aid of heat, 0.5 gm. being dissolved in approximately 20 cc. of water just prior to its use. This amount was injected into the jugular vein and oral administration was accomplished by dripping the solution into the animal's mouth so that none was lost.

Results.

On a standard balanced diet, the meat of which contained about 250 mg. of creatine, it was found that the animals excreted constant amounts of nitrogen and creatinine and only a trace of creatine appeared in the urine. The animals were then placed on a diet consisting of one egg, and cracker meal and lard in sufficient amounts to approximate their daily calorie requirement. This diet contained only traces of creatine and creatinine and the protein content was about the equivalent of the daily nitrogen loss during fasting. With this diet there was a prompt decrease in the urinary metabolites to a new level. With this marked decrease in nitrogen there was a definite reduction in the amount of creatinine excreted. These observations remained constant during the period of 3 weeks.

During the next 7 weeks the same diet was maintained and creatine hydrate (500 mg.) was administered daily. It was given by mouth to one animal and to the other intravenously. Since subsequent results did not show a difference in these two animals and because of the similarity of the two experiments, distinction need not be made between them. During this period of 7 weeks, with daily administration of creatine significant change did not occur in the excretion of creatinine by either animal. The increase in excretion of creatine was slight but definite the 1st day after administration. There was a gradual increase during the first 2 weeks, after which the excretion of creatine was constant; about 60 per cent of the injected material and about 50 per cent of the fed material was recovered unchanged in the urine.

In view of Benedict and Osterberg's experiments in which they fed casein to their dogs, I considered that this substance might

have some specific effect on the conversion of creatine to creatinine. Accordingly 10 gm. of casein daily were added to the diet but over a period of 2 weeks there was no significant change in the excretion of creatinine. After the 2nd day of casein feeding there appeared a definite drop in the creatine, but further decrease was not recorded the following days. One animal did not eat all of its food during the 2nd week and at this time the excretion of creatine returned to its former level. Practically all of the added nitrogen of the casein was retained during this period.

For the next 5 weeks 100 gm. of casein were added daily to the diet. The animal that had not taken all its food the previous week continued to take only small portions during the following 2 weeks. The other animal consumed its food each day. Within 2 days this animal showed marked retention of creatine and a suggestive rise in the excretion of creatinine. There was definite retention of nitrogen and the animal began to gain weight rapidly. In the succeeding weeks the retention of nitrogen was not quite so marked and the animal continued to gain weight. It was not until the end of the 2nd week that the creatinine increased markedly, although irregular excretion was noted earlier. For the 5th week of this period both animals showed an increase of 45 per cent in the creatinine of the urine. It should be noted that the animal not taking all of its food did not show these changes until it began to eat almost all of its food.

An attempt was then made to substitute 100 gm. of glucose for the casein of the previous period with the hope of maintaining the increasing weight of the animals without the increased nitrogenous metabolism. After the 1st week the animals could not be induced to take this large amount of carbohydrate, so that the results in the 2nd week are complicated by partial fasting of the animals. However, the excretion of creatinine continued at its high level for this period and the excretion of creatine increased to its former level at the end of the 1st week. Both animals lost weight during the 2nd week and it may be that the creatinuria was the result of their abstinence from food, since this was not observed during the 1st week.

After the animals were returned to the diet free from creatine and creatinine, the original levels of excretion of nitrogen and creatinine returned by the end of the 1st week. The excretion

of creatine was considerably increased so that practically the entire amount of creatine administered each day was recovered in the urine (Tables I and II).

TABLE I.
*Elimination of Nitrogen, Creatinine, and Creatine after Intravenous Injection of Creatine.**

Weight at end of period.	Daily average for 7 day periods.			Comment.
	Total N.	Creatinine	Creatine (expressed as creatinine).	
<i>kg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	
13 7	2752	330	35	
13 7	2703	367	27	
13 7	2458	359	29	
13.6	2724	349	75	Intravenous injection of 500 mg. creatine hydrate daily throughout rest of experiment.
13.6	2990	363	132	
13.2	3202	360	250	
13.2	3479	337	203	
13.3	2792	344	231	
13.2	2187	361	219	
13.1	2687	331	276	
13.1	2756	334	189	10 gm. casein added to diet.
13.2	2818	316	199	10 " " " " "
13.8	8561	377	96	100 " " " " "
14.6	9636	369	130	100 " " " " "
15.1	9857	428	18	100 " " " " "
15.5	10266	426	89	100 " " " " "
16 1	11138	521	28	100 " " " " "
15.9	4366	475	111	100 " glucose " " "
15.5	2624	524	184	100 " " " " " (not all food taken).
15.2	3430	474	358	No additions to diet.
15.0	2686	363	323	" " " "

* A male dog weighing 14.0 kilos received daily for several weeks the following diet (containing 6.65 gm. of nitrogen): cracker meal 121 gm., lean beef heart 121 gm., lard 22 gm., bone ash 11 gm. The average daily urinary excretion for 8 days was total nitrogen, 5585 mg., creatinine 390 mg., creatine 37 mg. The animal now weighed 13.8 kilos and received the following diet (containing 2.62 gm. of nitrogen) throughout the rest of the experiment: cracker meal 78 gm., one egg, lard 50 gm., bone ash 10 gm.

TABLE II.
*Elimination of Nitrogen, Creatinine, and Creatine after Oral
 Ingestion of Creatine.**

Weight at end of period.	Daily average for 7 day periods.			Comment.
	Total N.	Creatinine	Creatine (expressed as creati- nine).	
<i>kg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	
10.2	2712	238	20	
10.2	2870	241	22	
10.3	2174	253	21	
10.4	2328	260	118	Oral administration of 500 mg. creatine hydrate daily through- out rest of experiment.
10.3	2518	261	189	
10.0	2858	265	197	
10.1	2625	279	112	
10.2	2104	279	200	
10.2	2186	267	195	
10.2	2668	260	196	
10.2	2579	255	151	10 gm. casein added to diet.
10.1	3183	251	294	10 " " " " " (not all food taken).
9.9	4552	250	165	100 gm. casein added to diet (little food taken).
9.3	7796	264	224	100 gm. casein added to diet (not all food taken).
10.2	7162	270	74	100 gm. casein added to diet (al- most all food taken).
11.0	10935	291	61	100 gm. casein added to diet (all food taken).
11.6	11058	379	73	100 gm. casein added to diet.
12.0	4624	345	60	100 " glucose " " "
10.8	2746	390	227	100 " " " " " (very little food taken).
10.5	1967	270	178	No additions to diet.
9.7	2943	239	389	" " " "

* A male dog weighing 10.8 kilos received daily for several weeks the following diet (containing 5.55 gm. of nitrogen): cracker meal 101 gm., lean beef heart 101 gm., lard 18.5 gm., bone ash 9.5 gm. The average daily urinary excretion for 8 days was total nitrogen 3906 mg., creatinine 300 mg., creatine 50 mg. The animal weighed 10.2 kilos and received the following diet (containing 2.44 gm. of nitrogen) throughout the rest of the experiment: cracker meal 68 gm., one egg, lard 40 gm., bone ash 10 gm.

Comment.

It should be noted that male dogs were used in this experiment. Both animals were well nourished and muscular but neither was excessively fat. Both animals had been on diets containing considerable meat and were excreting small amounts of creatine. It is quite possible that different quantitative results might have been obtained with animals in which the creatine in the body had opportunity to decrease because of preliminary long continued creatine-free diets.

A marked difference was not obtained in the oral and intravenous administration of creatine except that a slightly greater amount of creatine was excreted following intravenous administration. Since this amount was so slight, its appearance in the urine is probably due to the plethora of creatine in the blood immediately following injection and would not be found when the creatine was more gradually absorbed from the gastrointestinal tract. Changes in creatine within the intestine must be very slight and may be disregarded; the utilization, conversion, or destruction of creatine must be sought within the organism.

The failure to obtain evidence of the conversion of creatine to creatinine during 9 weeks in which creatine was injected and a low protein diet was maintained affords ample evidence that the conversion of creatine to creatinine is not a direct function of the content of creatine in the body. The fact that one animal by refusing its high protein diet delayed the appearance of extra creatinine until 13 weeks after the administration of creatine was begun adds further proof, so it would appear that under certain conditions of metabolism the formation of creatinine is limited without regard to the quantity of creatine ingested. Definite storage of creatine took place during the first 2 weeks creatine was administered, but after that period a reasonably steady state was established in which 60 per cent of the injected creatine and 50 per cent of the fed creatine appeared unchanged in the urine. In view of this apparent balance it seems unlikely that the portions which are not accounted for could continue to be stored in the body, particularly since gain in body weight was not recorded. The amount of creatine lost may have been converted to creatinine and replaced or spared the original precursors of this substance, or,

as Benedict and Osterberg suggested, paths of metabolism of creatine not yielding creatinine may account for this loss of creatine. If this is true, an example of dissociation of the metabolism of creatine is apparent, since one type of metabolism may continue in the complete absence of the other.

There was a marked decrease in the recovery of creatine during the period of increased feeding of protein. With the retention of protein and the increase in body weight it might be considered that this creatine has also been stored, but it does not seem likely that creatine is stored in the same sense that protein is stored. Opposed to this view is the fact that 2 weeks after the retention begins creatinine increases in the urine and subsequent weeks show additional increases of creatinine. It would seem that the retained creatine is caught in a slow metabolic process which takes at least 2 weeks to convert creatine to creatinine. At the height of the excretion of creatinine in these experiments 43 and 34 per cent of the administered creatine appeared as creatinine, or 70 and 60 per cent of the creatine usually found in the urine appeared as creatinine. Since these experiments were discontinued before a constant level of excretion of creatinine was reached, it is possible that larger amounts of creatinine would have been found in the succeeding weeks.

It is extremely difficult exactly to correlate the observed changes in creatine and creatinine metabolism with the alterations produced in the protein content of the diet. Creatinine did not appear to increase in the urine until 2 weeks after the inception of the high protein diet and the subsequent increase in the total nitrogen of the urine. The increased elimination of creatinine continued 3 weeks after the discontinuation of the high protein diet. The body weight of the animals increased with the inception of the increased feeding, so that one would be as justified in attributing the changes produced to alterations in body weight as to alterations in protein metabolism. In these experiments the increased body weight was the result of increased administration of protein.

SUMMARY.

With a creatine-free diet low in protein, excretion of creatinine was not altered by daily feeding or by daily intravenous adminis-

tration of creatine in amounts comparable to the daily excretion of creatinine. Considerable amounts of the administered creatine were recovered unchanged in the urine. The addition of large amounts of protein (100 gm. of casein) to the diet greatly reduced the amount of creatine recovered in the urine. After about 2 weeks of high protein feeding and administration of creatine the excretion of creatinine began to increase and in some cases remained increased for a few weeks after the high protein diet was discontinued. This seems to be direct evidence of the conversion of creatine to creatinine and is also indicative of the relation of this conversion to the existing level of the metabolism of protein.

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A STUDY OF CREATINE METABOLISM IN THE NEPHRECTOMIZED WHITE RAT.*

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Numerous papers have been written on the fate of creatine in the organism, but no complete creatine balance has been obtained after oral or parenteral administration. The factors that must be investigated in order to trace administered creatine are (1) its excretion in the urine, (2) its partial conversion to creatinine, and (3) its deposition in the tissues; in addition, there is always a portion of this creatine which cannot be accounted for by any of our present methods. It is assumed by many workers that this "lost" creatine has undergone some anabolic change.

Hunter (1) has discussed the factors which are responsible for the excretion of creatine and creatinine under various experimental conditions. The storage and the metabolism of administered creatine present a problem which is poorly understood. It was first demonstrated by Folin (2) that the body can metabolize small quantities of ingested creatine so that it could not be accounted for in any way. Benedict and Osterberg (3) and Rose, Ellis, and Helming (4) have shown that small quantities of creatine, fed daily, can be handled by dog and by man without any trace of creatine or extra creatinine appearing in the urine for an appreciable period of time. Chanutin (5) has shown that the creatine content of the tissues of the white rat is not appreciably affected by the prolonged administration of a 2.6 per cent creatine diet. It has been found that in order to increase the creatine content of the tissues, it is necessary to increase the per cent of creatine in the diet (6). Obviously, creatine can be utilized or

* Part of the expenses of this investigation was defrayed by a grant from the funds of the Research Committee of the University of Virginia.

destroyed by the body within very wide limits before any storage in the tissues occurs. *One of the most outstanding facts in creatine-creatinine metabolism is the failure to account fully for administered creatine.*

The present experiments were undertaken with the view of eliminating the main pathway of creatine and creatinine excretion and thus, possibly, of learning more about the problem of creatine balance. A study was made of the effect of the injection of creatine on the tissues of nephrectomized rats, that had been fed on normal and creatine diets.

The general procedure in the care of the white rat and in the analysis of the tissues and the blood has been described in a previous paper (6). The experimental animals were fed up to the time of nephrectomy. The operation was performed under ether anesthesia by making a midline incision, and, after ligating the blood vessels and ureters, excising the two kidneys. The average time for this operation was about 7 minutes. Precautions were taken to keep the intestines warm and to prevent any unnecessary damage to the tissues. Immediately after the operation, creatine was injected subcutaneously. Because of the relative insolubility of creatine, it was found necessary to dissolve the largest quantity of material used (300 mg.) for injection in 7.5 cc. of water, and this same amount of water was used, both for the controls and for all creatine-injected animals. The subcutaneous injection was made in the back, about the level of the kidneys. At the time of death the creatine and water seemed to have been completely absorbed. The experimental animals were given no food after the operation, but were allowed water *ad libitum*. The animals were killed from 46 to 48 hours after the nephrectomy. As a rule, the healthy, mature white rat will survive this operation for about 70 hours, although occasionally animals have been encountered which were on the verge of coma after 48 hours. The skin used for analysis was obtained from the side opposite to that injected. The hair was clipped as close as possible with scissors, and adhering subcutaneous fascia was removed.

*Effect of Creatine Injection on the Creatine Content of
Nephrectomized Rats.*

The results of these experiments are summarized in Table I.

The percentage changes in the creatine content of the *brain* and

testes are relatively small. There is comparatively little change from the normal creatine concentration of these organs after the injection of 50 and 100 mg. of creatine. The first increase is noted after the injection of 200 mg. of creatine; a very slight further increase which represents the maximum storage in these organs is noted after the administration of 300 mg. The maximum percentage increases are 27.6 per cent for the brain, and 14.5 per cent for the testes.

The *heart* manifests no change until 100 mg. of creatine have been injected. The creatine concentration is increased markedly after larger quantities were administered. The maximum percentage increase in the creatine concentration of the heart was 61.4 per cent, attained after the injection of 300 mg. of creatine. It should be pointed out that despite an apparently large percentage increase in small organs, the actual amount of creatine stored must necessarily be small.

A survey of the literature concerning the distribution of creatine in the organism shows a complete neglect of the *skin* as a possible creatine-containing tissue. It was surprising, then, to find that rat skin contains about 0.110 per cent of creatine. This tissue, like the brain, testes, and heart, showed no appreciable percentage change in creatine concentration until 100 mg. had been injected. A maximum increase of 100 per cent was noted after the injection of 300 mg. of creatine.

The *liver* appears to be the most sensitive of all the organs in its response to creatine injection, for simple nephrectomy alone causes a 100 per cent increase in its creatine concentration. The increase in the creatine concentration of the liver seems to be roughly proportional to the amount injected. A maximum creatine concentration of 0.396 per cent is obtained in contrast to the normal concentration of 0.030 per cent. It seems logical to assume that any organ which responds as readily to the oral or parenteral administration of creatine, must have some function in the metabolism of this material. However, the present evidence available seems to point to a lack of function of the liver in creatine metabolism. It is quite possible that the ready increase in creatine concentration in the liver is indicative of an effort on the part of the organism to excrete this material by way of the alimentary tract.

It is generally believed that the *muscle* is the most important

Liver, total solids.	Minimum.	29.3	27.9	27.0	28.1	26.6	27.1	27.0	27.2	26.6	24.0	24.9	26.0
	Maximum.	32.3	28.7	30.9	30.2	29.5	28.9	29.1	28.9	29.3	28.9	28.6	29.5
	Average.	30.8	28.2	28.3	29.1	28.1	28.0	28.1	28.2	27.9	27.8	27.1	28.0
	No. of rats.	5	5	12	7	11	6	12	9	12	13	10	10
Liver, corrected.	Minimum.	0.025	0.209	0.041	0.158	0.117	0.138	0.132	0.210	0.245	0.196	0.308	0.333
	Maximum.	0.036	0.308	0.080	0.270	0.171	0.327	0.308	0.420	0.310	0.475	0.529	0.502
	Average.	0.031	0.268	0.065	0.218	0.137	0.257	0.219	0.315	0.275	0.341	0.396	0.431
	No. of rats.	5	5	12	7	11	6	9	9	12	13	10	10
Skin.	Minimum.		0.134	0.106	0.145	0.102	0.148	0.144	0.168	0.164	0.172	0.205	0.233
	Maximum.		0.152	0.137	0.169	0.133	0.190	0.176	0.233	0.194	0.265	0.229	0.325
	Average.		0.145	0.119	0.156	0.120	0.168	0.156	0.196	0.174	0.210	0.222	0.266
	No. of rats.		5	6	7	5	6	6	8	5	13	5	10
Muscle.	Minimum.	0.468	0.514	0.432	0.497	0.418	0.488	0.470	0.512	0.452	0.452	0.491	0.517
	Maximum.	0.526	0.544	0.515	0.534	0.495	0.586	0.525	0.624	0.511	0.605	0.559	0.641
	Average.	0.497	0.528	0.474	0.516	0.457	0.539	0.495	0.536	0.492	0.544	0.513	0.589
	No. of rats.	13	5	9	6	11	5	12	9	10	9	12	11
Muscle, total solids.	Minimum.	24.1	21.3	20.2	21.0	19.9	20.9	20.7	20.1	20.0	19.8	20.6	20.3
	Maximum.	25.4	23.0	23.8	23.6	23.3	22.7	23.2	23.9	23.0	23.2	24.6	23.8
	Average.	24.6	22.4	22.2	22.8	21.3	21.8	21.9	21.6	21.4	21.4	22.5	22.4
	No. of rats.	14	5	9	6	11	5	12	9	10	9	12	11
Muscle, corrected.	Minimum.	0.469	0.580	0.511	0.550	0.502	0.586	0.541	0.569	0.551	0.571	0.532	0.615
	Maximum.	0.530	0.616	0.549	0.595	0.572	0.666	0.590	0.655	0.605	0.690	0.610	0.710
	Average.	0.505	0.591	0.537	0.566	0.539	0.631	0.569	0.622	0.580	0.638	0.573	0.680
	No. of rats.	13	5	9	6	11	5	12	9	10	9	12	11
Weight of rat.	Average.	222	264	268	207	264	239	271	254	284	266	257	271

tissue concerned in creatine metabolism. This conclusion is based on the high percentage and actual content of creatine in muscle. This tissue has been studied almost exclusively by investigators in an effort to trace administered creatine. Under any given set of conditions, we have found the muscle to be very resistant to a percentage change in its creatine concentration. In Table I, it is seen that the concentration of muscle creatine is not changed appreciably as the result of the injection of 100, 200, or 300 mg. of creatine. This response of the muscle differs from any other tissue in that the maximum percentage storage is as great after 100 mg. as after 300 mg. of creatine. It is obvious, therefore, that any attempt to study creatine metabolism by means of muscle creatine determinations must take into consideration the difficulty of obtaining changes in creatine concentration in this tissue by any set experimental procedure.

Effect of Creatine Feeding and Creatine Injection.

Previous work from this laboratory has demonstrated that creatine feeding can raise the creatine concentration of various tissues. An attempt has been made to obtain a cumulative effect by creatine feeding and injection combined with nephrectomy, in the hope that a maximum saturation of the creatine reservoirs could be obtained.

The experimental animals were fed a 10 per cent creatine diet for 5 days. At the end of this time both kidneys were removed, and at the time of operation the stomach was examined to be sure that the animals had been eating the food. After 2 days of fasting, the animals were killed.

The results of the analyses on various tissues are set forth in Table I. The data show quite clearly that creatine feeding increases the creatine content of the tissues above the level of nephrectomized rats fed on a normal diet. An attempt to correlate the actual and the theoretical increases in the creatine concentration of various tissues was made, but no relationship whatsoever could be found.

By comparison of the data for the control and creatine-fed animals, as shown in Table I, the only noteworthy change noted after the injection of water alone was the increase in the creatine

content of the liver. The rise noted in the liver is to be expected, since it has been demonstrated in normal animals after the ingestion of creatine (5, 7). There was also a definite increase in the creatine concentration of the remaining organs analyzed.

After the injection of various amounts of creatine, the values obtained illustrate the tendency of the creatine concentration to rise. The data show that the increases obtained are in no way comparable to the amounts of creatine injected. Despite the tremendous increase in the creatine content of the organism due to creatine ingestion and injection the animal seems to metabolize a very large proportion of the administered creatine. The values for creatine in the tissues of this group of animals are not large enough to explain the fate of administered creatine as due to storage alone.

It may be well to point out some of the results that were obtained after an animal had been subjected to an experimental "saturation" with creatine. In these creatine-fed animals injected with 300 mg. of creatine, the average percentage concentration of muscle was 0.660 which represents an increase of 47 per cent over the normal (0.450). The highest individual creatine concentration was 0.710 per cent. The liver as usual shows the greatest difference from normal; an average creatine concentration of 0.431 was obtained which contrasts markedly with the normal of 0.030 per cent. An increase of 120 per cent over the normal creatine concentration (0.119) was noted for the skin. This increase is an appreciable one because of the proportion of the body weight that the skin comprises. The brain shows very little change from the normal. The heart and the testes show increases of 82 and 32 per cent respectively. We believe that these percentage increases represent the maximum storage of creatine in the tissues of the organism.

Studies on the Blood of Nephrectomized Rats Injected with Creatine.

Non-Protein Nitrogen.—The determination of non-protein nitrogen was undertaken to study the effect of nephrectomy and injection of creatine on the accumulation of nitrogenous bodies in the blood (Table II). As a result of nephrectomy alone the non-protein nitrogen increases from 43 to 239 mg. per 100 cc. of blood. The injection of varying amounts of creatine into

TABLE II.
Effect of Creatine Injection on the Blood of Nephrectomized Rats Fed Normal and Creatine Diets.
 Figures are expressed in mg. per 100 cc. of blood.

Diet.....	Control.	Creatine.	Control.	Creatine.	Control.	Creatine.	Control.	Creatine.	Control.	Creatine.	Control.	Creatine.	Control.	Creatine.
	Control incision.													
Amount injected into nephrectomized animal.														
	None.	7.5 cc. water.			50 mg. creatine.			100 mg. creatine			200 mg. creatine.			300 mg. creatine.
		212	227	224	226	177	220	187	228	214	234			
Non-protein N.	Minimum.	181	315	300	320	253	375	343	303	276	329			
	Maximum.	279	239	263	274	240	274	265	274	251	278			
	Average.	5	11	6	7	3	6	9	7	6	6			
	No. of rats.													
Creatine.	Minimum.	3.3	10.2	26.4	24.6	43.0	29.6	59.2	62.3	80	129			
	Maximum.	5.3	18.9	79.1	38.8	89.8	67.9	93.2	85.4	133	201			
	Average.	4.3	14.4	51.0	31.4	63.0	42.1	78.0	74.0	112	164			
	No. of rats.	11	8	7	8	3	12	7	9	12	11			
Preformed creatinine.	Minimum.	1.1	7.5	10.9	8.8	10.9	7.4	12.3	9.9	12.7	9.8	12.9		
	Maximum.	2.4	11.5	15.8	15.2	14.3	14.2	16.8	14.6	17.2	14.5	23.1		
	Average.	1.5	9.6	13.6	12.9	12.5	10.9	14.6	12.1	15.2	11.9	16.6		
	No. of rats.	11	11	6	9	3	12	8	10	13	10	11		
Sugar.	Minimum.	76	81	95	107	100	80	83	84	95	103	96		
	Maximum.	143	126	143	152	165	149	100	135	131	145	118		
	Average.	103	111	109	125	125	114	95	115	111	120	105		
	No. of rats.	11	12	5	9	3	12	4	10	7	10	4		

nephrectomized rats does not increase the non-protein nitrogen to an appreciable extent.

Creatine and Creatinine.—Clinical and experimental studies of nephritis show a marked accumulation of creatine and creatinine in the blood. The present studies afford an opportunity for testing the ability of the organism to convert creatine to creatinine, if the blood analyses of these constituents may be taken as an indication of this conversion. The readiness of the tissues to dehydrate creatine rapidly is well demonstrated, but this change proceeds only to a definite limit, beyond which a further change is very difficult.

The results of total nephrectomy alone (Table II) demonstrate a greater percentage increase in creatinine (700 per cent) than in creatine (360 per cent). The injection of 50 mg. of creatine affected the blood creatinine to the same extent that an injection of 300 mg. did. The blood creatine rose in proportion to the increasing dosage of creatine, and under such circumstances, one might expect a corresponding increase in the creatinine concentration of the blood, but apparently the limit of creatine conversion to creatinine is dependent on factors other than the amount of creatine in the blood stream.

Chanutin and Silvette (6) have shown that creatine fed to normal rats causes a marked increase in blood creatine, but only a slight increase in blood creatinine. In nephrectomized animals which had been fed a 10 per cent creatine diet (Table II), the blood creatinine is 40 per cent higher than that of animals similarly treated but fed a normal diet. The injection of 50 and 100 mg. of creatine does not increase the blood creatinine level appreciably above the control; however, larger amounts (200 and 300 mg.) cause a comparatively large increase. After the injection of 300 mg. of creatine to these animals the blood creatinine rises to a figure 1300 per cent above the normal.

In the light of these results, it is impossible to correlate the creatine concentration of the blood with the accompanying creatinine concentration. It seems certain that an organism exposed to a condition of almost complete creatine saturation, as it was under these experimental conditions, should display the maximum ability to convert creatine to creatinine. As a result of these experiments it is felt that creatinine accumulation in the

blood of the nephritic is not altogether a question of poor excretion. The experimental animal is able to convert creatine to its anhydride to a very limited extent and beyond that point the creatine concentration has no effect.

DISCUSSION.

The results which have been obtained in this paper seem to indicate that the fate of creatine in the body is still an unsettled problem. It has been possible to demonstrate a marked but limited conversion of creatine to creatinine by means of the blood studies. Furthermore a storage of creatine in various organs has been demonstrated. There still remains a portion of administered creatine which cannot be accounted for by our method of analysis.

The difficulty in tracing ingested creatine was first pointed out by Folin (2). Small doses of creatine literally disappeared, since no trace of creatine, extra creatinine, or an increase in urinary nitrogen could be obtained. Experiments by Rose and Dimmitt (8), Benedict and Osterberg (3), Chanutin (9), and Rose, Ellis, and Helming (4), performed on man and dog further substantiated the evidence that it was impossible to trace the metabolism of creatine by urinary studies. The studies of these and other investigators have led Hunter ((1), p. 163) to conclude that "creatinine is not subject in metabolism to any catabolic 'destruction.'" The only change which it has yet been definitely proved to undergo is its conversion into creatinine. The creatine which disappears after ingestion must therefore have been retained in some form within the organism." Chanutin (5) attempted to trace the ingested creatine in the tissues of rats and could find a relatively slight increase only in the livers of these animals. Further studies (5, 7) in mice and rats showed an increase in the creatine concentration of muscle and liver and other organs provided large amounts of creatine were fed. In other words, small doses of ingested creatine seem to "disappear" according to tissue studies and only a small amount of this creatine is recovered when comparatively large doses are fed. In the light of such evidence we must conclude that much of the ingested creatine is either destroyed or converted into other materials. It is assumed of course that only a limited amount of administered creatine is excreted by the kidneys.

Myers and Fine (10) were able to demonstrate a rise of 5 per cent in the creatine concentration of rabbit muscle after continued daily subcutaneous injections of creatine. From an analysis of several hundred rat muscles we believe that a 5 per cent variation is, in the long run, within the experimental variation of creatine determination in tissues. Further evidence to support the current idea that muscle can easily store creatine is derived from the work of Folin and Denis (11). They were able to demonstrate variable but marked increases in the muscle of cats several hours after creatine had been introduced into the small intestine. This is not surprising since we know how readily many absorbed products are temporarily stored in the muscle shortly after a meal.

TABLE III.

Absolute Increase of Creatine in Tissues of Nephrectomized Rats After Creatine Injection.

Creatine injected.	Brain, weight 1.6 gm.	Heart, weight 0.65 gm.	Liver, weight 10.0 gm.	Testes, weight 2.5 gm.	Skin, weight 0.18 × body weight.	Muscle, weight 0.44 × body weight.	Blood, volume 14 cc.	Total.	
mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	per cent
0	0	0	0	0	0	0	0	0	0
50	0	0	6.7	0.2	1.0	-10.0	2.2	-0.8	-1.6
100	0.2	0.1	14.3	0.5	19.0	42.0	3.7	79.8	79.8
200	0.7	0.4	19.6	1.1	32.0	90.0	8.3	152.1	76.1
300	0.8	0.7	30.6	1.5	45.0	15.0	17.0	110.6	36.9

It is clear from available evidence that excess creatine storage in tissues is not easily attained over a period of time, and furthermore the actual amount stored above the normal concentration that can be accounted for is comparatively small.

In an effort to study the fate of creatine injected into nephrectomized rats, calculations of the actual amounts of creatine deposited in tissues comprising 75 per cent of the body weight have been made. The skeleton, which comprises about 11 per cent of the rat's weight, is a negligible factor in creatine storage, hence brings the actual true percentage of creatine deposition closer to a complete balance. The intestines, spleen, spinal cord, lungs, fat, and other glands and tissues which have been omitted from our analyses are known to be very low in creatine concentration and

not liable to be considered as important reservoirs for creatine storage. It is felt therefore that the tissues which can be responsible for the storage of 90 to 95 per cent of administered creatine have been analyzed.

The results of these calculations have been summarized in Table III. Donaldson's (12) standards for the percentage of body weight of muscle, skin, and blood have been used. The arbitrary weights for brain, liver, testes, and heart were obtained by averaging the weights of a large number of tissues in our experimental animals. In order to make the results comparable it was found necessary to use the tissues and blood of nephrectomized animals injected with 7.5 cc. of water as controls.¹

A rough calculation of the creatine content of the nephrectomized rat weighing 260 gm. yields a figure of approximately 715 mg. After the injection of 50 mg. of creatine subcutaneously the percentage recovery is for all purposes negative. This simply shows that the injection of approximately 7 per cent of the total creatine content of the rat can escape detection by our method of analysis. After the injection of 100 and 200 mg. of creatine we are able to detect 79.8 and 76.1 per cent, respectively, of the amount injected. The muscle, skin, and liver are the principle reservoirs. On the other hand the injection of 300 mg. of creatine leads only to a very small recovery of 36.9 per cent. In this case the skin and liver carry the burden of the storage while the muscle plays only a minor rôle. It is difficult to reconcile these results since one would certainly expect the muscle to be responsible for a great deal more of the stored creatine in this series of animals. However, it must be noted that the percentage change of creatine in muscle varies very little after the injection of 100, 200, and 300 mg. of creatine.

¹ The method of calculation is as follows: 44 per cent of the average weight of the nephrectomized animals in any one group was used as an index of the actual weight of the muscle. The average weight of the muscle of the control nephrectomized rats was calculated to be 118 gm. ($268 \text{ gm.} \times 44 = 118$). Since the percentage of the creatine in the muscle of these animals was 0.537, we find that the actual creatine content of muscle is 635 mg. per 118 gm. of muscle. By application of this same calculation to the muscle of the 300 mg. series, for example, an increase of 15 mg. over the control is obtained ($650 - 635 \text{ mg.}$).

As a result of our analyses, it seems certain that a very large part of injected creatine cannot be accounted for. It should be pointed out that not only creatine but also creatinine is represented, if present, in the figures given as creatine values for the tissues. In view of the high blood creatinine it seems quite possible that tissues might have an appreciable creatinine concentration. The partial conversion of creatine to creatinine has been demonstrated by numerous investigators. According to Benedict the "metabolism of creatine takes place through two or more different paths, only one of which yields creatinine." Our experiments appear to demonstrate the conversion of creatine to creatinine and a limited storage of the administered creatine. The creatine that cannot be accounted for in these experiments must have undergone either an anabolic or catabolic change. In the experiments reported in this paper, the possibility of anabolism as a means of accounting for the missing creatine seems improbable. We believe that the evidence in this and other investigations points toward a breaking down of a part of the administered creatine to products that have not been isolated or studied. The fact that neither ammonia nor urea are increased in the body tissues and fluids after the administration of creatine cannot be considered as positive evidence that this substance is not catabolized.

There is still another possibility to be considered in connection with the portion of the creatine that has disappeared. The intestines may play a part in the excretion of creatine or its by-products. It should be pointed out that there are practically no references in the literature to the possible products of creatine that may be formed in the intestines, and to the amount of such products that may be excreted. The liver does not seem to play any rôle of importance in creatine metabolism (1). If this is true, the marked accumulation of creatine in the liver after the ingestion or injection of creatine may very well be an indication of a hepatic pathway for the excretion of creatine into the intestine. It has been shown, furthermore, that the increase of creatine in the liver is only a transitory one. It is well known that there are intestinal bacteria that are able to break down creatine, and under such circumstances it is quite possible to overlook any evidence of creatine excretion by the intestinal tract.

Studies from this laboratory seem to indicate a marked resistance

of muscle towards any unusually high storage of creatine. Under any particular experimental condition it appears to be difficult to increase the storage of excess creatine after a level is reached despite any marked increase in creatine administration. Nevertheless the creatine saturation point seems to be quite a variable one. The creatine content of muscle changes with fasting, creatine feeding, creatine injection, nephrectomy, and with combinations of any of these factors. We must conclude from the evidence of resistance on the one hand and flexibility of creatine storage on the other that the mechanism of catabolism and anabolism of creatine in the muscle is poorly understood.

It is generally conceded that creatinine arises from creatine. Much discussion has centered about the idea of creatinine excretion being an indication of a general endogenous (Folin) or muscular metabolism (Shaffer). From our evidence we feel that the brain, heart, and testes play only a minor rôle in creatine-creatinine metabolism. The "extra" creatine of the liver is known to be retained only for short periods. Little is known about the state of creatine in the skin but there is every reason to suppose that creatine is stored temporarily just as in the case of the liver. An analysis of the creatine content of the control nephrectomized rat shows that about 90 per cent of the creatine of the organism is found in the muscular tissue. If the blood creatinine can be taken as an index of creatinine formation in the body, it is seen that this constituent roughly parallels the percentage change of creatine in the muscle of nephrectomized animals. The relationship noted here is further evidence to support the idea that the muscle is preeminently prominent in the formation of creatinine.

This work has led us to continue certain phases of this problem. Experiments have been begun to study the creatine concentration of the entire carcass in an effort to trace ingested and injected creatine. An effort is being made to trace the possible excretion of creatine by the liver into the intestine. We are furthermore interested in the stability of creatine storage in the skin.

SUMMARY.

The effect of varying amounts of subcutaneously injected creatine upon the creatine content of the brain, heart, testes, skin, liver, and muscle has been studied in completely nephrectomized

rats that had been fed normal and creatine-containing diets. Percentage increases in creatine content were noted in all tissues studied, particularly in the liver, skin, and muscle. Calculations of the creatine balance after creatine administration show a very positive loss which leads to the belief that creatine is destroyed by the organism. Evidence is presented to show a relationship between muscle creatine and creatinine formation.

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THE NATURE AND IDENTITY OF WHEAT GLUTENIN.*

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INTRODUCTION.

The gluten of wheat flour has always been of outstanding importance and interest to the wheat industry in general and to the cereal chemist in particular. Osborne's (1907) well founded conclusion that gluten consists primarily of two distinct and individual proteins, glutenin and gliadin, and his characterization of these two proteins has for many years served as the foundation for most modern thought as to their nature and respective individualities.

Of these two proteins glutenin has within recent years come into special prominence, due largely to evidence obtained by Sharp and Gortner (1923) indicating that glutenin is the protein that is solely responsible for variations among the colloidal properties of glutes from different flours, and that these variations may in turn be important causes of differences among the bread-making characteristics of the flours in question.

Both proteins have been frequently isolated and purified by various investigators, and their constitution and properties have been studied by such methods as have been available. The nitrogen distribution method of Van Slyke has been chiefly relied upon to indicate chemical constitution. There is ample justification for the belief that the values yielded by Van Slyke's procedure are, for the most part, far from being absolute, even though they may be highly informative. Results of protein analyses by this procedure are at best strictly comparable only when the same manipulative conditions are maintained in all instances.

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Various workers have had occasion to prepare the two gluten proteins, glutenin and gliadin, in as pure form as possible, and have subjected their preparations to Van Slyke analysis. In almost all instances the methods of preparation and purification of these proteins have either followed closely or have been based upon the methods originally used and described by Osborne. On comparison of different analyses of the same protein, it is at once noticeable that there is greater concordance among the results of analyses of gliadin than is correspondingly true with glutenin. One cannot escape the conviction that the chemical identity of the former is far more definitely established than that of the latter.

TABLE I.

Values for Amide Nitrogen and for Arginine Nitrogen in Various Gliadin Preparations.

Analyst.	Source of flour.	Amide N.	Arginine N.
		<i>per cent</i>	<i>per cent</i>
Osborne (1924).	Unknown.	24 5	
Van Slyke (1911).	"	25 5	5 71
Osborne, <i>et al.</i> (1915)	"	24 6	5 45
Blish (1916)	Spring wheat.	26 1	4 55
" "	Soft "	25 9	4 47
" and Sandstedt (1924).	Hard winter.	26 4	
Cross and Swain (1924).	Idaho.	26 8	4 86
" " " "	Patent.	26 4	5 06
" " " "	Club.	26 4	5 21
" " " "	Fortyfold.	26 2	4 78
Hoffman and Gortner (1927).		25 9	5 29
Dill and Alsberg (1925)		26 2	

In considering variations among results of Van Slyke analyses of different preparations of the same protein, attention may appropriately be confined to the values for amide nitrogen and for arginine nitrogen. These two constituents are determined simply and directly, and their estimation may be regarded as subject to less error than is involved in the estimation of the other Van Slyke units. Comparative data of this nature for gliadin and glutenin are shown in Tables I and II, respectively.

Inspection of data in Tables I and II discloses far greater variability among analyses for glutenin than for gliadin. In the case

of gliadin, all values for amide nitrogen fall within extremes of 24.5 to 26.8 per cent, with 7 of the 12 values falling close to 26. The extremes for arginine nitrogen in gliadin are 4.47 and 6.38 per cent, with most values reasonably concordant. Among the glutenin samples, on the other hand, the range of values for amide nitrogen is 12.4 to 18.8 per cent, and for arginine nitrogen the range is 8.18 to 12.94 per cent.

The situation becomes all the more conspicuous when the comparison of variability among gliadin preparations with that of glutenin preparations is confined to samples all of which were prepared in the same laboratory, opportunity for such a compari-

TABLE II.
*Values for Amide Nitrogen and for Arginine Nitrogen in Various
Glutenin Preparations.*

Analyst.	Source of flour.	Amide N.	Arginine N.
		<i>per cent</i>	<i>per cent</i>
Osborne (1924).	Unknown.	18 8	
Blish (1916).	Spring wheat.	16 5	9.69
" "	Soft "	16 2	9 27
Cross and Swain (1924).	Idaho.	15 6	10 10
" " " "	Patent.	16 0	8 18
" " " "	Club.	14 2	9 23
" " " "	Fortyfold.	13 1	12 94
Hoffman and Gortner (1927).	Patent.	13 6	11 96
Larmour (1927).	"	14 8	10 90
Blish (unpublished).	Hard winter.	13 2	12 50
" "	" "	12 4	11 20

son being afforded by the data of Cross and Swain (1924). They selected four different types of wheat and made carefully prepared preparations of gliadin and glutenin, respectively, from each lot. Their extreme values for amide nitrogen among the four samples of gliadin were 26.2 and 26.8, and values for arginine nitrogen fell between the limits 4.78 and 5.21. Among the four glutenin samples, on the other hand, values for amide nitrogen range from 13.1 to 15.6, and those for arginine nitrogen run from 8.18 to 12.94, the latter values incidentally being not only the extremes for the one laboratory, but for all workers whose data appear in Table II.

Cross and Swain (1924) regard their data as evidence that gliadins from different wheats are identical, and such a conclusion is obviously justified. However, they conclude that glutenins from different wheats are also identical, a decision for which there is far less justification. The data for glutenin would seem rather to indicate that one of two things is true: (1) either glutenins from different wheats are not identical, or (2) methods for the preparation and purification of glutenin are uncertain and unreliable, and the true chemical nature, identity, and individuality of this protein have not been satisfactorily established. This communication is a preliminary report of certain experiments bearing upon the latter possibility.

Glutenin, according to Osborne's (1924) characterization, is that portion of the wheat flour protein that is left after complete removal of all protein material soluble in dilute neutral salt solutions and in 50 to 70 per cent alcohol. It is readily soluble in dilute alkali, and this reagent has invariably been used as the initial solvent or dispersing agent in the isolation of the glutenin, which is later precipitated from the filtered extract by neutralization with acid. That treating protein with alkali tends to produce certain alterations in the protein molecule is well known. Among the effects known to be possible are liberation of ammonia, destruction of cystine, splitting of arginine into ornithine and urea, and racemization. The rate at which these alterations occur depends of course upon factors such as concentration of OH ions, temperature, and time of exposure. In the preparation of the cereal glutelins, however, the alkali has been of such dilution, and other factors have been such that serious alteration of the protein molecule has not generally been suspected. That this possibility should be taken more seriously becomes evident when Hoffman (1925) reports that cystine can no longer be isolated from human hair after it has received such a mild treatment as washing with hot 1 per cent sodium carbonate solution. In the present studies one of the first items to be considered has been the effect of different concentrations of alkali used in extracting the glutenin, upon the nitrogen distribution in the resulting product after its complete hydrolysis in strong acid.

In the first series of experiments herewith reported, glutenin preparations were isolated from two different lots of the same

flour, the flour having previously been extracted with 5 per cent K_2SO_4 solution (followed by distilled water) to remove albumin and globulin, and dried *in vacuo* at a low temperature. A 40 gm. portion of this flour was extracted for 20 minutes, with constant shaking, with 300 cc. of $N/60$ NaOH. An equal portion was similarly extracted with 300 cc. of $0.2 N$ NaOH. Each lot was then made up to 1 liter with 95 per cent methyl alcohol, according to the procedure recommended by Blish and Sandstedt (1924). This gives a final alcoholic concentration from which the starch rapidly settles, permitting ease of filtration, and the alcohol also retains the gliadin in solution when the extract is neutralized in order to precipitate the glutenin. After filtration, a suitable portion of each extract was neutralized with HCl to the point of optimum flocculation of the glutenin. After flocculation and

TABLE III.
*Total, Amide, and Basic Nitrogen in Glutenins A and B,
after Complete Hydrolysis.*

Preparation.	Strength of alkali used in initial extraction of glutenin.	Total N in hydrolysate.	Amide N.	Basic N.
		gm.	per cent	per cent
A	$N/60$	0.1393	21.4	9.70
B	$N/5$	0.1093	18.1	13.1

settling, the clear supernatant liquids were poured off, and the precipitates were washed repeatedly by decantation with 65 per cent methyl alcohol solutions containing respectively the calculated concentrations of NaCl as formed by neutralization of the original extracts. Each preparation was then completely hydrolyzed with strong HCl, and Hausmann numbers were determined, with results as presented in Table III. These glutenins as prepared by the weaker and the stronger alkali are designated, respectively, as Glutenins A and B.

Data in Table III show a marked difference in the percentages of both amide and basic nitrogen as produced by different alkali concentrations used for dispersing and extracting the glutenins originally, the higher alkali concentration causing a marked decrease in amide nitrogen and a very pronounced increase in basic or diamino acid nitrogen.

These experiments were repeated, the same procedure being followed in all respects, except that the glutenin was precipitated by neutralizing the alkaline extracts with CO_2 instead of HCl . The results are shown in Table IV.

Although the actual values in Table IV are slightly different from those in Table III, the comparative effects of the different alkali concentrations are shown to be the same as in Table III.

The next experiment involved preliminary extraction of the same flour with 0.05 N and 0.25 N NaOH solutions, respectively, followed by dilution of the extracts with methyl alcohol, as before, and precipitation of glutenin from the filtered extracts by neutralization with CO_2 . In this instance, however, instead of merely washing the precipitated glutenins by decantation, they were

TABLE IV.

Total, Amide, and Basic Nitrogen in Glutenins A and B, the Glutenins Having Been Precipitated from Their Alkaline Extracts by Neutralization with Carbon Dioxide.

Preparation.	Strength of alkali used in initial extraction of glutenin.	Total N in hydrolysate.	Amide N.	Basic N.
		gm.	per cent	per cent
A	N/60	0.205	20.3	11.51
B	N/5	0.125	16.9	14.75

redissolved and reprecipitated several times from dilute alcoholic alkali in order to remove all possibility of adhering or occluded gliadin. The preparation that was originally dispersed by 0.05 N alkali showed, after hydrolysis, an amide nitrogen content of 15.9 per cent, whereas a value of only 12.1 per cent was found in the case where 0.25 N alkali had been the preliminary dispersing agent. These values are of a sufficiently lower order than those secured in the preceding experiments to indicate that the several redispersions and reprecipitations used for purification purposes caused a substantial lowering of both values for amide nitrogen, comparative values remaining in the same order, however. The values for basic nitrogen were more than twice as high as in the preceding experiments, being 22.1 per cent for the preparation involving the weaker NaOH , and 24.5 per cent for the stronger. The same

tendency persists as to correlation between strength of alkali and per cent of basic nitrogen. These results are typical of many experiments subsequently performed and involving concentration of NaOH as the sole variable.

In view of this situation it was considered desirable to attempt the preparation of glutenin under conditions avoiding exposure to alkaline reaction at any stage of the procedure. At first, numerous attempts based upon acid dispersion failed because of inherent difficulties in physical manipulation. Glutenin apparently is dispersed in very dilute acid with difficulty and only under certain conditions. When thus dispersed, its ultimate particles are so large, due either to large aggregates or to high degree of hydration, or both, that it will not pass through a filter to permit elimination of starch and other solid foreign material. Furthermore it was difficult to find a method for coagulating it from acid dispersion without also precipitating gliadin in physical combination. Definite and uniform preparations were, however, finally secured by the following procedure: Crude gluten is prepared from flour by kneading the flour-water dough under tap water in the usual manner. This gluten is finely macerated and placed in a large volume of very dilute acetic acid. After standing overnight it is thoroughly dispersed, giving a somewhat viscous solution with starch and other solid foreign material in suspension. Filtration being impossible, the solution is diluted with methyl alcohol until an alcoholic concentration of 65 to 70 per cent is obtained. The dilute alcoholic solution is then passed slowly through a Sharples supercentrifuge, whereby starch and other suspended matter are removed, giving a highly opalescent sol. When this solution is neutralized to a pH slightly below 7, by slowly stirring in N NaOH solution (but never allowing the material to become alkaline), a heavy, gelatinous protein precipitate comes down rapidly. The gliadin remains in the alcoholic solution, and is at once decanted off. The precipitate may be redispersed in dilute acetic acid, and reprecipitated after the solution is again diluted with methyl alcohol. After two or three such treatments, followed each time by washing with water, the gliadin is completely eliminated as shown by absence of protein in the supernatant liquid. The precipitated glutenin is then dried in the usual manner with alcohol and ether. It was noted that a satisfactory

separation of glutenin could not be obtained when ethyl alcohol was substituted for methyl alcohol. The reason for this is doubtless associated with the known greater dehydrating power of methyl alcohol.

Preliminary observations and studies of several samples of glutenin prepared in this manner indicate that it differs both physically and chemically from products whose preparation has involved more or less prolonged exposure to alkaline reaction at intervals during the process. There are both physical and chemical differences. When freshly precipitated, the new protein is far more coherent and gelatinous than are preparations precipitated from alkaline extracts, the latter being decidedly flocculent and non-coherent. The new protein bears a close resemblance physically to the original crude gluten, lacking only in elasticity and toughness.

The nitrogen content is probably close to 17.50 per cent. One sample showed 17.4 per cent nitrogen, as corrected to a moisture- and ash-free basis. It is difficult completely to eliminate the starch, and all samples thus far have shown traces of starch or dextrin to the extent that they do not give a water-clear solution in alkali until this minute quantity of foreign matter has settled out. The amide nitrogen after acid hydrolysis has in all cases run close to 22 per cent of the total nitrogen, this value being decidedly higher than any recorded for glutenin preparations involving alkali (see Table II), and being only about 4 per cent below the average of recorded values for gliadin (see Table I). Percentages of arginine nitrogen were estimated in these preparations by the procedure recently suggested by Plimmer (1925); that is to say, the entire filtrate from the amide nitrogen determination was used for prolonged boiling with strong NaOH, instead of first precipitating the diamino acids with phosphotungstic acid as in the customary procedure. This method used gives somewhat higher results than the usual procedure, since it probably accounts for some arginine that would escape precipitation by phosphotungstic acid. The arginine values for all preparations of the new glutenin ran close to 9 per cent of the total nitrogen. This indicates a lower percentage of arginine in the new than in the old preparations.

That the new preparation is in no way physically contaminated

with gliadin is evidenced by the fact that the dry and finely powdered material yielded no trace of protein upon prolonged extraction with 70 per cent alcohol.

Preparations of glutenin resembling those made according to the conventional method (see Table II) may readily be produced from the new protein by dissolving it in alkali, and later precipitating by neutralizing the alkaline solution. Thus three samples of the new preparation were dispersed in solutions of 0.02 N, 0.1 N, and 0.2 N NaOH, respectively. After standing overnight the solutions were diluted with alcohol and were neutralized by treatment with CO_2 . The precipitates were washed, hydrolyzed, and tested for amide nitrogen, yielding 16.85 per cent, 12.53 per cent, and 10.32 per cent respectively, the per cent of amide nitrogen being as usual inversely proportional to the concentration of alkali.

The ultimate particles, or molecules, as the case may be, of the new protein are apparently of greater size and complexity than those of glutenin as ordinarily prepared by methods involving extraction with and exposure to alkali. This was indicated by examining the protein remaining in solution after precipitation of the glutenin by neutralization of the alkaline solutions in the immediately preceding experiments. The chemical constitution of this protein remaining in the supernatant liquid differs from that of glutenin prepared either by the alkali or acid dispersion method. Upon hydrolysis it yields approximately 25 per cent of its total nitrogen as amide nitrogen, and its percentage of arginine nitrogen runs about 7 to 8 per cent. These values are close to the figures obtained with pure gliadin. Whether or not it actually is identical with gliadin is now under investigation. The fact that it cannot be removed by alcohol alone, but is split off only after treatment with alkali strongly suggests that the protein that has customarily been regarded as glutenin does not exist as such in the original flour, but is rather a product of the action of alkali upon a larger and more complex protein body.

When glutenin is precipitated from alkaline alcoholic extracts of flour or gluten, not only does the composition of the precipitate vary with the strength of the alkali, but the *amount* of protein precipitated varies inversely as the strength of the alkali, as has been shown by Blish (1926). Thus the quantity of protein re-

maining in the supernatant liquid will vary also. It is of interest, however, that experimental evidence thus far obtained in this laboratory indicates that although the *precipitated glutenin* varies both in amount and composition, under these conditions, the protein left in the supernatant liquid varies in amount only, and not in composition. This adds support to the idea that the gluten of wheat flour contains, in addition to gliadin, a protein body that is more complex than either gliadin or what is generally regarded as glutenin. The effect of alkali upon this larger protein entity is to split off protein resembling gliadin in properties thus far studied, the amount split off depending upon the strength of the alkali, or conditions of exposure thereto. The remaining fraction represents glutenin as it has ordinarily been prepared and studied, and the nature of the alkali treatment will largely determine both its quantity and its chemical constitution.

In view of the inherent difficulties involved in definitely establishing the identity or individuality of proteins, and since the work thus far completed is preliminary in character, it would be hazardous at this stage to attempt to classify the new preparation as a distinct and individual protein. Some nine or ten samples have been prepared. They agree within a reasonable factor of error both as to per cent of amide nitrogen (averaging about 22 per cent) and arginine nitrogen (averaging about 9 per cent). This indicates a definite and consistently uniform composition.

It is of course improbable that any protein may be isolated from biological material and subjected to the customary methods of purification without undergoing either physical or chemical alteration, or both. As to the new glutenin preparations herewith described, there is substantial assurance that far less alteration has occurred than is ordinarily the case with preparations made according to the principles usually and heretofore employed.

Whatever may be the nature or importance of the new "glutenin," these experiments clearly indicate an irreversible alteration in the chemical structure of a considerable portion of flour or gluten protein when dispersed in an alkaline medium, regardless of alkali concentration. It is likely that this factor has also influenced the composition and properties of glutelins that have been prepared from other cereals as well. Kondo and Hayashi (1926) after experimenting with rice glutelin have recently stated

their conviction that it is not possible to prepare individual proteins whose chemical identities are established beyond all doubt, especially where temporary solution in alkali is involved. They find that rice glutelin is very susceptible to alteration even when the alkali is very dilute, and express the belief that the best that may be expected under such circumstances is a protein that is well defined and reproducible. Their experiences, together with those of the present writers, serve as a substantial confirmation of the statement that appears in Osborne's monograph on "The Vegetable Proteins" (1924 edition) when he said in speaking of the glutelins: "Very little that is definite has been learned respecting these proteins and until they have been further studied by modern methods it is hopeless to discuss them further."

SUMMARY.

1. Glutenin, as prepared by customary methods involving extraction with or temporary solution in alkali, is a product resulting from an irreversible alteration by the action of alkali on a more complex protein body.

2. Both yield and chemical constitution of glutenin prepared by the usual methods will vary with the concentration of alkali.

3. A new "glutenin" has been prepared by a procedure in which exposure to alkali is avoided at all stages. It differs from the usual glutenin both in physical properties and in chemical constitution.

4. It is probable that some irreversible alteration occurs when any protein material is dispersed in alkaline solution, regardless of the concentration of alkali.

5. There is occasion for further intensive investigation of the nature of the nitrogenous material of wheat and flour, and of the other cereals as well, and such investigation will doubtless lead to a substantial revision of present day ideas as to the true character of this protein or group of proteins, as the case may be. This situation applies with special force to the so called cereal "glutelins."

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TRYPTOPHANE AND GROWTH.

II. GROWTH UPON A TRYPTOPHANE-DEFICIENT BASAL DIET SUPPLEMENTED WITH TRYPTOPHANE DERIVATIVES.*

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For a number of years we have been interested in the possibility of replacing the "indispensable" amino acids of the diet by synthetic compounds, and of determining the influence of the latter upon the growth process. Such studies, as pointed out frequently by us in former papers, appear to afford the possibility of learning much concerning the types of chemical reactions which the animal organism is capable of accomplishing. In this and the following paper we are presenting the results of experiments involving the replacement of tryptophane.

We have undertaken first a study of the influence exerted by substituent radicals introduced in the amino or carboxyl group, but without altering the remainder of the molecule. For this purpose four derivatives, namely, acetyl-, benzoyl-, and methylene-tryptophane,¹ and tryptophane ethyl ester hydrochloride, have been prepared and employed in the dietary in place of equimolecular amounts of the free amino acid.

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† The experimental data in this paper are taken from a thesis submitted by Clarence P. Berg in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Physiological Chemistry in the Graduate School of the University of Illinois.

¹ We have assumed that the formaldehyde condensation product is a hydrated methylenetryptophane (*cf.* Homer, 1913), and have referred to it as such throughout this paper.

The literature indicates that the biological introduction of acetyl groups into amino acids may occur fairly frequently during the process of metabolism. Thus Knoop (1910, 1911) discovered that α -amino- γ -phenylbutyric acid may undergo acetylation in the dog. Neubauer and Warburg (1910-11) observed a similar reaction following the perfusion of the surviving liver with phenylaminoacetic acid. Dakin (1911), upon administering inactive *p*-methylphenylalanine to an alcaptonuric, isolated from the urine a small amount of the dextro-acetyl derivative. Furthermore, Sherwin and his associates (Cerecedo and Sherwin, 1923-24, 1924-25; and Muenzen, Cerecedo, and Sherwin, 1926) have shown that *o*-, *m*-, and *p*-aminophenylacetic acids are detoxicated by acetylation in the rabbit. The para form of the acid is disposed of in a similar fashion in man. Other illustrations of similar reactions are available in the literature.

On the other hand, the removal of substituent groups by the animal organism frequently appears to be a more difficult process. Thus Magnus-Levy (1907) injected subcutaneously a number of benzoylated amino acids, and in practically every case recovered the original substance in the urine in an amount large enough to cause him to believe that the compound had not undergone cleavage. Shiple and Sherwin (1922) fed phenylacetyl derivatives of glycocoll, alanine, leucine, glutamine, glutamic acid, asparagine, aspartic acid, and ornithine. These investigators report: "In every case results showed that when the amino group is phenylacetylated, complete or even partial catabolism of the amino-acid is prevented. . . ." Cystine derivatives, however, appear to be exceptions as Sherwin, Shiple, and Rose (1927) state: "Phenylacetyl and acetyl radicals are ineffectual for blocking and exert no influence on the oxidation of cystine sulfur. . . ." Benzoylation of cystine inhibits oxidation completely. Lewis, Updegraff, and McGinty (1924) have clearly shown that the injection of dibenzoylcystine does not lead to an increased output of oxidized sulfur. They attribute the finding by Epstein and Bookman (1914) of a moderate increase in hippuric acid excretion following the feeding of benzoylalanine to partial hydrolysis of the compound in the alimentary tract.

Most of the experiments outlined above have involved the administration of compounds containing the phenyl group. The well known stability of the benzene ring in the animal organism may account for the inhibition in oxidation. It does not follow necessarily, therefore, that derivatives not containing the phenyl radical would be as resistant to change. Furthermore, it is conceivable that the administration of a compound in comparatively small quantities at frequent intervals might lead to better utilization than would follow the feeding or injection of a single large dose.

Very little information is available as to the effect of substituent groups in the tryptophane molecule. In a preliminary report,

Jackson (1928) states that neither the betaine of tryptophane nor the formaldehyde condensation product is able to replace the amino acid for growth purposes.

EXPERIMENTAL.

All of the compounds used in our experiments were synthesized in these laboratories. The acetyl and benzoyl derivatives, and the ethyl ester hydrochloride have not been described heretofore. The formaldehyde condensation product was made according to the method of Homer (1913).

For the preparation of *acetyltryptophane*, 1 gm. of the amino acid was dissolved in 10 cc. of approximately N sodium hydroxide. To this solution were added in 1 cc. portions, 3 cc. of redistilled acetic anhydride, each addition being followed by vigorous shaking for several minutes. The cloudiness noted upon adding the first fraction cleared when the subsequent additions were made. After all of the acetic anhydride had dissolved, the solution was kept in a hot room at 35–40°. Upon standing 2 to 3 hours, a precipitate of white, glistening platelets separated. The latter were filtered off, washed several times with 5 to 10 cc. portions of cold water, and suspended in 30 cc. of N hydrochloric acid to remove any unreacted tryptophane which may have been present. The material was again filtered off, washed free of chlorides with cold distilled water, and dried in a vacuum oven at 80°. The yield amounted to 0.77 gm. A second run, in which 8 gm. of tryptophane were employed, yielded 5.98 gm. The derivative shrinks at 205° and melts at 206–207° (uncorrected) with apparent decomposition. It responds to the Hopkins-Cole glyoxylic acid test but produces no color with bromine water. It is practically insoluble in water and in dilute acid, but is readily soluble in ether, alcohol, and alkali. The neutral equivalent was found to be 247.7, and the nitrogen percentage 11.35. The theoretical values are 246.1 and 11.38, respectively.

The synthesis of *benzoyltryptophane* was accomplished as follows: 1 gm. of tryptophane was dissolved in 60 cc. of water together with 6.5 gm. of sodium bicarbonate. 1.5 cc. of benzoylchloride were added, a little at a time, with vigorous shaking over a period of an hour. The solution was allowed to stand an additional 2 hours, or until the odor of benzoylchloride had disappeared. The mixture

of benzoic acid and benzoyltryptophane was then precipitated by adding 5 per cent sulfuric acid until the solution became distinctly acid to Congo red. Precipitation was completed by cooling in the ice box for 24 hours, after which the solids were filtered off, washed with water until free of chlorides, and dried in the air at 40°. The benzoic acid was removed from the crystalline mixture by two or three extractions with cold benzene. The final residue was air-dried. The yields obtained in several runs varied from 0.5 to 0.84 gm. The crystals are glistening white platelets. If precipitated too rapidly from a concentrated solution, the mixture of benzoic acid and benzoyltryptophane is apt to separate as a gummy mass which will usually solidify if kept in the refrigerator for a day or two. The crude crystalline compound melts at 98–103° (uncorrected) after softening at 91°. It may be reprecipitated by dissolving in 95 per cent alcohol and adding water, or by dissolving in hot 33 per cent alcohol and cooling in the ice box for 24 to 48 hours. Recrystallization leads to a loss of approximately one-third of the material. Upon concentrating the mother liquors *in vacuo* a second crop of crystals may be obtained. Occasionally the recrystallized product has a pink or purplish tint. The pure derivative softens at 100° and melts at 104–105° (uncorrected). Our preparation showed a neutral equivalent of 310.8 and yielded 9.13 per cent of nitrogen. The theoretical values are 308.1 and 9.09 per cent, respectively.

According to Miss Homer (1913), *methylenetryptophane* as synthesized by her procedure and dried *in vacuo* contains 2 molecules of water of crystallization. Analysis of our preparation confirmed this statement. Our product showed a neutral equivalent of 253.8 and a nitrogen content of 11.00 per cent. The theoretical values for $C_{12}H_{12}N_2O_2 \cdot 2H_2O$ are 252.1 and 11.11, respectively. The yields obtained from 1 gm. portions of tryptophane varied from 1.0 to 1.11 gm.

The method used in the synthesis of *tryptophane ethyl ester hydrochloride* was similar to the one employed by Abderhalden and Kempe (1907) in preparing the methyl ester hydrochloride. For this purpose, 8 gm. of tryptophane were suspended in 80 cc. of absolute ethyl alcohol in a flask fitted with a 2-hole rubber stopper carrying a calcium chloride drying bulb and a delivery tube. The flask was placed in a freezing mixture, and the alcohol saturated

with dry hydrogen chloride gas. In an hour or two the bulk of the tryptophane had dissolved. After saturation was complete, the reaction mixture was allowed to warm to room temperature, whereupon all of the solid material dissolved. The excess hydrogen chloride was removed by evaporation practically to dryness *in vacuo*.² The residue was dissolved in a minimum amount of boiling absolute alcohol, and treated with hot ethyl acetate until flocculation began. Crystallization was completed by cooling overnight in the ice box. The precipitate was filtered off, washed twice with 10 cc. portions of ethyl acetate, and once with 10 cc. of absolute ethyl alcohol. After drying, the material was redissolved in hot absolute alcohol (20 cc. per gm.), and reprecipitated as before. The twice recrystallized product softened at 219° and melted at 221° (uncorrected) with decomposition. The yield amounted to 6.2 gm. The nitrogen content was found to be 10.29 per cent as compared with a theoretical value of 10.43 per cent.

Each of the above compounds was tested for its growth-promoting properties by being administered to young rats upon a tryptophane-deficient basal diet. The latter was composed of acid-hydrolyzed casein 14.7, cystine 0.3, dextrin 40, sucrose 15, lard 19, cod liver oil 5, salt mixture (Osborne and Mendel, 1919) 4, and agar 2 per cent, and was furnished *ad libitum*. The synthetic compounds were fed at 12 hour intervals (*cf.* Berg and Rose, 1929) in doses equivalent to 10 mg. of tryptophane, and were incorporated in the vitamin pills. Each of the latter contained 150 mg. of yeast, 75 mg. of dextrin, and enough water to make a stiff dough. Thus each rat received daily 300 mg. of yeast, and a quantity of the tryptophane derivative equivalent to 20 mg. of the amino acid. For comparison, control animals of the same litter received either no supplement in the vitamin pills or were furnished 10 mg. of tryptophane at intervals of 12 hours.

The results of the experiments are summarized in Charts I to IV. As will be observed, the animals which received the acetyl derivative or the ethyl ester hydrochloride grew at rates which are

² During the evaporation of the excess hydrogen chloride a red color appears, but this is completely removed during the subsequent reprecipitation. A like color is obtained when tryptophane stands in contact with air in a hydrochloric acid solution, or may be produced by treating a tryptophane solution with chlorine water.

quite comparable to those displayed by the controls which received tryptophane. On the other hand, the benzoyl and methylene derivatives showed no supplementing effects whatever. The rats which received these products lost weight rapidly until minimal levels had been attained, which subsequently were maintained with

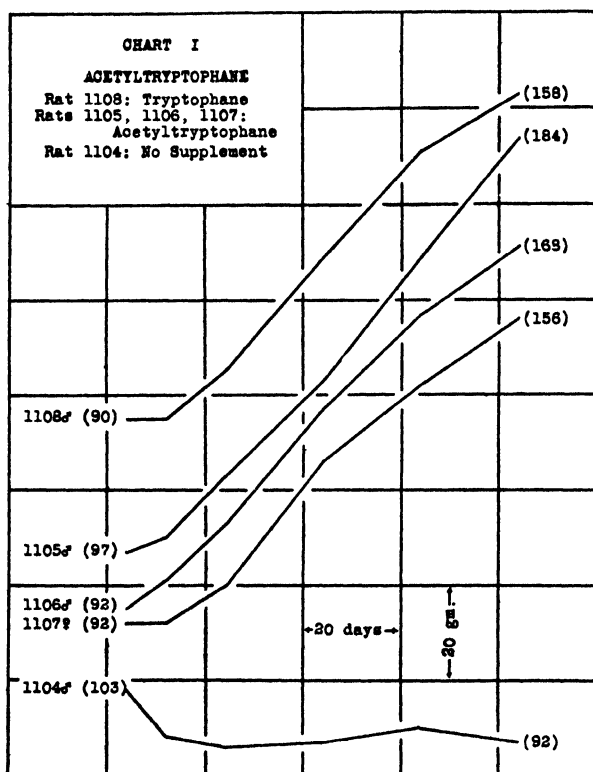


CHART I.

minor fluctuations. Despite the similarity in type of linkages in the acetyl and benzoyl derivatives, the compounds behaved quite differently in the organism. Evidently, benzoyl- and methylenetriptophane are not available sources of the free amino acid.

Attempts were made to determine whether the two active

derivatives are hydrolyzed in the alimentary tract under the influence of digestive enzymes. Inasmuch as the substitution products fail to respond to the bromine water test characteristic of free tryptophane, it appeared to be a simple matter to show whether the

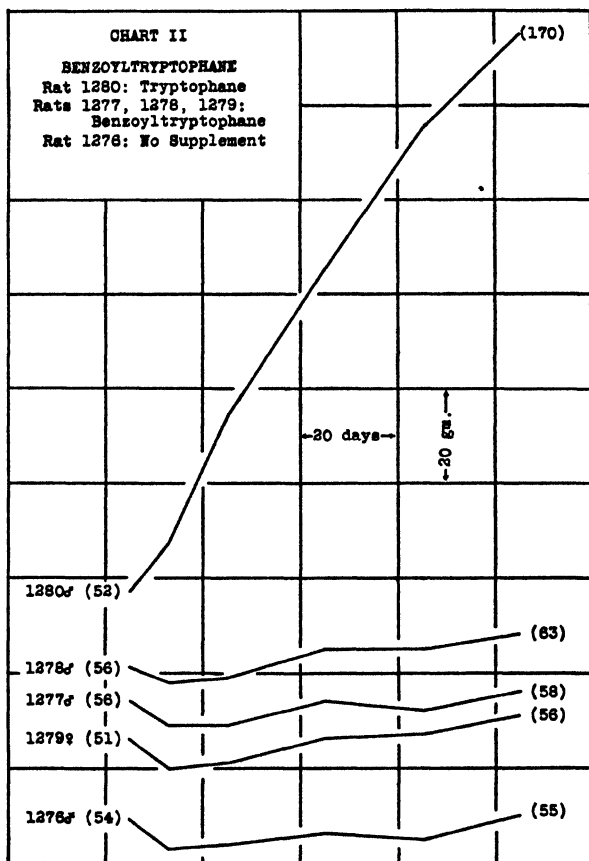


CHART II.

amino acid is liberated by intestinal enzymolysis. In the case of the ethyl ester hydrochloride there is no doubt of the fact that such liberation of free tryptophane occurs. After digesting the derivative with commercial pancreatic enzymes, or with freshly prepared

toluene-water extracts of rat intestines, strong tests for free tryptophane were secured. Evidently, therefore, the efficiency of the ester as a growth-promoter may be explained on the basis of the liberation of the free amino acid preceding absorption.

On the other hand, the results of similar experiments with the acetyltryptophane indicate that very little enzymatic hydrolysis occurs. *In vitro* tests show that after portions of the compound

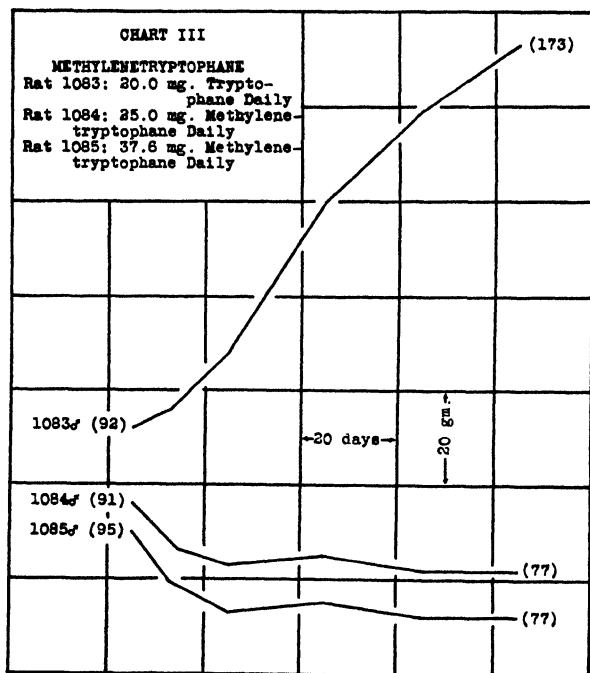


CHART III.

have remained in contact with the enzymes for 12 hours the resulting solutions may give a very faint color with bromine. Several commercial enzyme preparations, as well as freshly prepared toluene-water extracts, were employed in the tests. Of these, the greatest effect was induced by "holadin."³ Even with this the

* Fairchild Brothers and Foster.

color intensity at the end of 12 hour digestion periods indicated that the degree of hydrolysis was very slight.

While a positive statement as to the method of utilization of acetyltryptophane would not be justified by the information

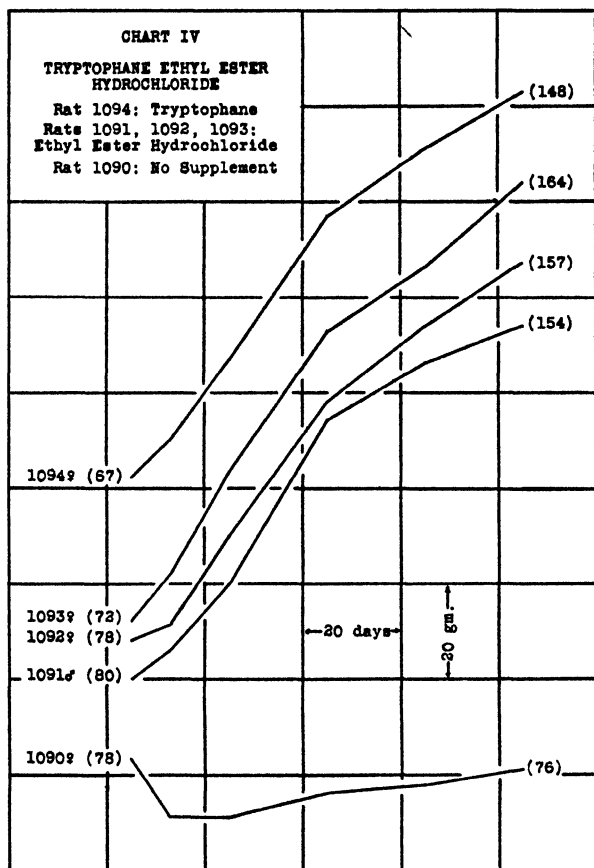


CHART IV.

available, it seems likely, in the light of our experiments, that the compound undergoes deacetylation after, rather than before, absorption. The efficiency with which the derivative replaces free tryptophane for purposes of growth, and the slow rate at which it

undergoes hydrolysis under the influence of the alimentary enzymes, favor the view that cleavage of the material is largely a postabsorptive process. If this conception is correct, the experiments herein recorded provide an interesting illustration of reversal of the reaction of biological acetylation observed by Knoop (1911) and others. Furthermore, the strikingly different growth responses elicited by the introduction in the diet of the benzoyl and acetyl

TABLE I.

Growth upon a Tryptophane-Deficient Basal Diet Supplemented with Acetyltryptophane.

Rat No. and sex.	Average daily food consumption.	Average daily change in weight.	12 hr. supplement.
	<i>gm.</i>	<i>gm.</i>	
1104♂	4.0	-0.14	None.
1105♂	6.3	+1.09	12.1 mg. acetyltryptophane.
1106♂	6.5	+0.95	12.1 " "
1107♀	6.0	+0.80	12.1 " "
1108♂	5.9	+0.85	10 " tryptophane.

TABLE II.

Growth upon a Tryptophane-Deficient Basal Diet Supplemented with Benzoyltryptophane.

Rat No. and sex.	Average daily food consumption.	Average daily change in weight.	12 hr. supplement.
	<i>gm.</i>	<i>gm.</i>	
1276♂	3.2	+0.01	None.
1277♂	3.4	+0.03	15.5 mg. benzoyltryptophane.
1278♂	3.5	+0.09	15.5 " "
1279♀	3.2	+0.06	15.5 " "
1280♂	6.1	+1.48	10 " tryptophane.

derivatives, each of which contains the peptide-like linkage so readily severed in many other compounds, serve to emphasize anew how exceedingly exacting the organism is in its growth requirements.

In conclusion we call attention to the food consumption data summarized in Tables I to IV. In every instance the animals which grew ate more food than those which failed to grow, despite

the fact that the supplements were fed invariably in the vitamin pills where their presence could not influence the taste or texture of the basal ration. This is in accord with numerous observations (*cf.* Rose, 1928) indicating that rats upon deficient diets experience a loss of appetite, but that the latter is restored when the ration is rendered adequate by suitable supplementation.

TABLE III.

Growth upon a Tryptophane-Deficient Basal Diet Supplemented with Methylenetryptophane.

Rat No. and sex.	Average daily food consumption.	Average daily change in weight.	12 hr. supplement.
	gm.	gm.	
1084♂	4.5	-0.18	12.5 mg. methylenetryptophane.
1085♂	4.0	-0.23	18.8 " "
1083♂	6.8	+1.01	10 " tryptophane.

TABLE IV.

Growth upon a Tryptophane-Deficient Basal Diet Supplemented with Tryptophane Ethyl Ester Hydrochloride.

Rat No. and sex.	Average daily food consumption.	Average daily change in weight.	12 hr. supplement.
	gm.	gm.	
1090♀	4.0	-0.03	None.
1091♂	6.0	+0.93	13.2 mg. ester.
1092♀	7.2	+0.99	13.2 " "
1093♀	6.9	+1.15	13.2 " "
1094♀	6.8	+1.01	10 " tryptophane.

SUMMARY.

Four derivatives of tryptophane have been fed to rats upon tryptophane-deficient diets in order to determine whether the substances in question are capable of serving in place of the amino acid for purposes of growth. The results indicate that methylenetryptophane and benzoyltryptophane are unable to meet the requirements of the organism in lieu of the free amino acid. Evidently, the introduction of the substituent groups renders the resulting products useless from the nutritive standpoint.

On the other hand, acetyltryptophane and tryptophane ethyl ester hydrochloride are utilized for growth purposes just as satisfactorily as is free tryptophane. In the case of the ethyl ester hydrochloride it is quite likely that the compound undergoes hydrolysis in the alimentary tract. Indeed, experiments *in vitro* showed that enzymes can accomplish this change. With acetyltryptophane, however, evidence of *alimentary* deacetylation is less easily secured. Apparently, extracts of the intestinal mucosa of rats, as well as solutions of commercial enzymes, liberate the amino acid from its derivative very slowly. Inasmuch as the compound is utilized for growth as readily as is tryptophane itself, and under such conditions must be employed, at least in part, in the synthesis of tissue proteins, it appears probable that deacetylation occurs chiefly after the derivative has been absorbed from the intestine. If this be true, the experiments afford an illustration of biological deacetylation.

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TRYPTOPHANE AND GROWTH.

III. 3-INDOLEPROPIONIC ACID AND 3-INDOLEPYRUVIC ACID AS SUPPLEMENTING AGENTS IN DIETS DEFICIENT IN TRYPTOPHANE.*

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It has been known for a number of years that the animal organism possesses the power of synthesizing α -amino acids out of the corresponding α -hydroxy or α -ketonic acids. Thus Knoop and Kertess (1911) isolated from the urine the acetyl derivative of *d*-phenyl- α -aminobutyric acid following the administration to a dog of phenyl- α -ketobutyric acid. By means of perfusion experiments, Embden and Schmitz (1910, 1912) have shown that the ammonium salts of pyruvic, phenylpyruvic, and *p*-hydroxy-phenylpyruvic acids are transformed by the liver into alanine, phenylalanine, and tyrosine, respectively. They observed also the production of alanine from lactic acid. Using a similar method, Kondo (1912) has reported the synthesis of α -aminobutyric acid and norleucine from the corresponding ketonic acids.

More recently, Cox and Rose (1926) and Harrow and Sherwin (1926) have demonstrated the fact that for purposes of growth 4-imidazole lactic acid is capable of serving as a dietary component in place of histidine. The latter authors have shown that 4-imidazole pyruvic acid manifests a similar property. On the contrary, α -hydroxy- ϵ -aminocaproic acid (McGinty, Lewis, and Marvel, 1924-25), 3-indolelactic acid (Jackson, 1927), and β -dithiodilactic acid (Westerman and Rose, 1928) do not promote the growth of animals upon diets deficient in the corresponding amino acids, lysine, tryptophane, and cystine. It appears that if these synthetic hydroxy acids are transformed into the amino acids the rate of reaction is not sufficient to meet the demands of growth.

* This communication was presented in abstract before The Thirteenth International Physiological Congress at Boston, August, 1929.

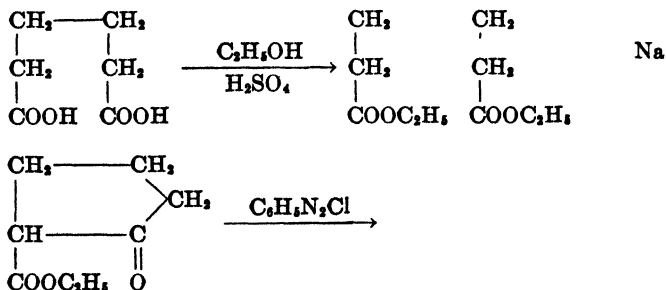
† The experimental data in this paper are taken from a thesis submitted by Clarence P. Berg in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Physiological Chemistry in the Graduate School of the University of Illinois.

Our interest in the nutritive behavior of tryptophane prompted us to determine whether the functions of this amino acid may be performed by 3-indolepropionic acid and 3-indolepyruvic acid. After the work upon the first of these products had been completed, there appeared a preliminary report of similar studies by Jackson (1928) in which the author states that 3-indolepropionic acid is not available for growth in place of tryptophane. Our data are entirely in accord with this conclusion. Indeed, the literature shows that all attempts to replace essential amino acids by synthetic compounds of analogous structures *in which the α -hydrogens are unsubstituted* have yielded negative results (*cf.* McGinty, Lewis, and Marvel, 1924-25; Cox and Rose, 1926; and Westerman and Rose, 1927). On the other hand, unpublished experiments of Heft and Sherwin (1926) are said to show that 3-indolepyruvic acid is also incapable of replacing tryptophane in the diet. With this statement we are entirely unable to agree. As will be demonstrated below, 3-indolepyruvic acid manifests a remarkable efficiency in substituting for tryptophane as a growth essential.¹

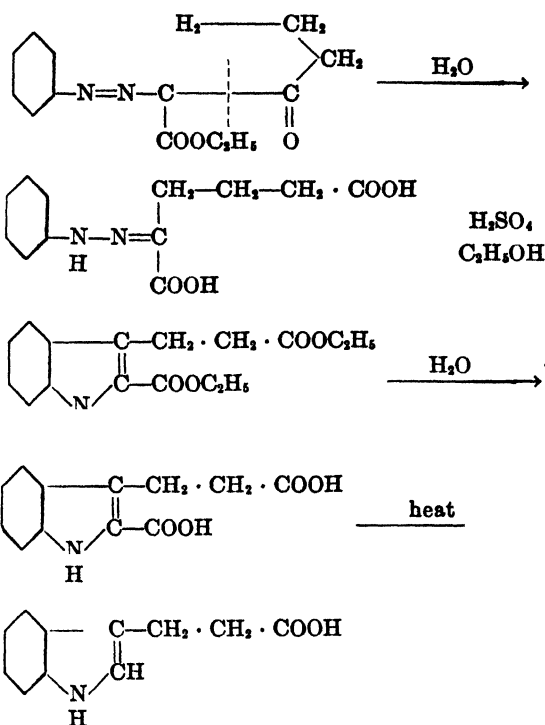
EXPERIMENTAL.

The two indole acids were prepared in these laboratories by the use of synthetic methods reported in the literature, except for slight modifications which we have found advantageous.

3-Indolepropionic acid was made from adipic acid by the steps indicated in the accompanying diagram.

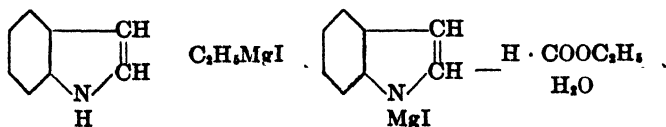


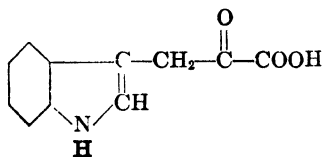
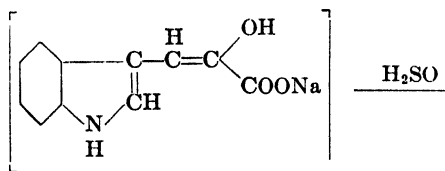
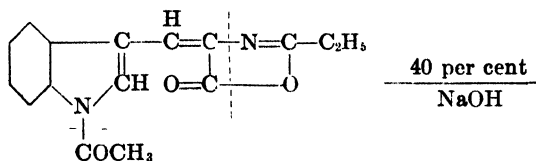
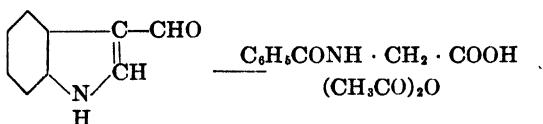
¹ A personal communication from Dr. R. W. Jackson indicates that he also has been able to demonstrate that 3-indolepyruvic acid can serve in place of tryptophane for purposes of growth.



Diethyladipate was prepared and condensed with itself to give ethyl-1,2-cyclopentanone carboxylate according to the method of van Rysselberge (1926). From this intermediate, 3-indolepropionic acid was synthesized according to the procedure of Kalb, Schweizer, and Schimpf (1926) as modified by Manske and Robinson (1927). The final product melted at 133–134°, had a neutral equivalent of 188.7, and contained 7.42 per cent of nitrogen. The theoretical values for the neutral equivalent and per cent of nitrogen are 189.1 and 7.41 respectively.

3-Indolepyruvic acid was synthesized from indole in the steps shown in the diagram which follows.





3-Indolealdehyde was prepared by Putochin's (1926) modification of the method of Majima and Kotake (1922) except that in place of anisole or benzene as a solvent for the preparation and use of the Grignard reagent di-*n*-butyl ether was substituted. This change in the procedure gave a product which was more easily purified. The 3-indolealdehyde was converted to 3-indolepyruvic acid according to the procedure of Ellinger and Matsuoka (1920). The final product was of a light buff color. When heated slowly it darkened at 198°, softened at 204°, and melted at 212°. The neutral equivalent was found to be 204.6, and the percentage of nitrogen was 6.97. The theoretical values are 203.1 and 6.89, respectively. Ellinger and Matsuoka do not report the melting point of the acid, but state that the *p*-nitrophenylhydrazone melts at 153–154°. The *p*-nitrophenylhydrazone of our material melted at this temperature also.

The compounds, prepared as described above, were tested for their growth-supporting ability by feeding them either separately

or incorporated in the tryptophane-deficient basal ration. The latter had the usual composition: acid-hydrolyzed casein 14.7, cystine 0.3, dextrin 40, sucrose 15, lard 19, cod liver oil 5, salt mixture (Osborne and Mendel, 1919) 4, and agar 2 per cent. It was always fed *ad libitum*. When the derivatives were incorporated in the food mixture, they supplanted equal weights of hydrolyzed casein.

Two series of feeding experiments were carried out with indolepropionic acid. In the first, the derivative was fed in the food mixture at a 0.37 per cent level, representing twice the molecular equivalent of tryptophane employed in the tryptophane control diet. Vitamin B was supplied in the form of pills composed of 200 mg. of yeast, 100 mg. of dextrin, and enough water to make a stiff dough. The pills were fed once daily. Two litters of six and seven rats respectively were used. In each litter, two rats received a diet in which 0.2 per cent of tryptophane replaced 0.2 per cent of the hydrolyzed casein, two rats were given no supplement, and the remaining animals were fed a ration in which 0.37 per cent of indolepropionic acid supplanted the corresponding percentage of casein digest. In the second series, the derivative was fed apart from the other food at a daily level of twice the molecular equivalent of 20 mg. of tryptophane. Two litters of four rats each were used. One rat from each litter received 10 mg. of tryptophane at 12 hour intervals, one rat was given no supplement, and the other two animals received 18.5 mg. of indolepropionic acid every 12 hours. The supplements were incorporated in the yeast pills, each of which contained 100 mg. of yeast, 50 mg. of dextrin, and enough water to make a compact mass.

One litter of six rats was employed in testing the growth-promoting ability of indolepyruvic acid. The animals were divided as follows: two were placed upon the usual tryptophane-deficient basal diet, and four upon a diet in which 0.02 per cent of the hydrolyzed casein had been supplanted by 0.02 per cent of tryptophane. Vitamin B was supplied in the form of pills composed of 250 mg. of yeast, 125 mg. of dextrin, and enough water to make a stiff dough. The pills were administered at 12 hour intervals. The object of feeding yeast at this high level and of including a small amount of tryptophane in the ration of some of the rats was to prevent the latter from losing weight and becoming

distinctly abnormal as the result of a tryptophane deficiency. It seemed likely that under the conditions employed the animals would remain more responsive to any supplementing action which the indolepyruvic acid might exert.

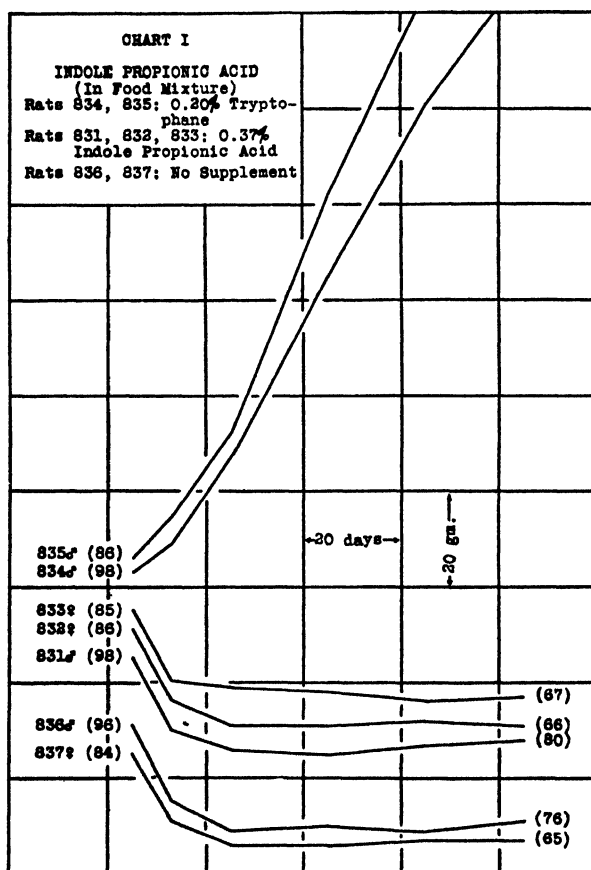


CHART I.

After 48 days upon the above diets, one of the two rats upon the ration devoid of added tryptophane received indolepyruvic acid in the food mixture at a level of 0.2 per cent. To the other animal the synthetic product was administered each day in the

vitamin pills in doses of 10 mg. every 12 hours. Likewise, two of the rats upon the 0.02 per cent tryptophane basal diet received indolepyruvic acid in the food mixture at a 0.2 per cent level, one was given 20 mg. of the compound each day in two doses in the vitamin pills, and one received no supplement whatever.

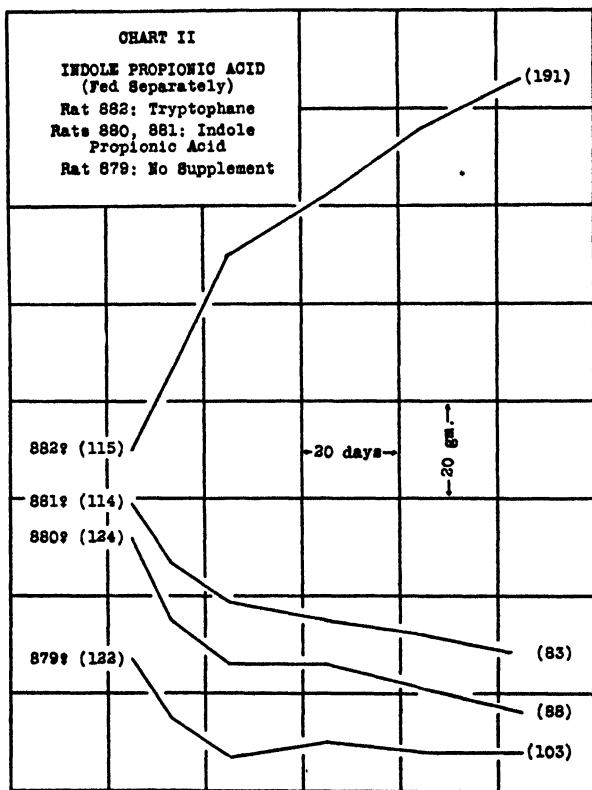


CHART II.

After administration of the derivative for 28 days, four of the rats were returned to their original diets. The other animal (Rat 1144) received for 12 days double the quantity of indolepyruvic acid, after which the compound was omitted from the food. Finally, for purposes of comparison the ration of each individual was sup-

plemented for 28 days with an amount of tryptophane equivalent to the indolepyruvic acid previously employed.

The results of the experiments are summarized in Charts I to III. In Charts I and II are reproduced the data secured with two of the four litters of rats employed in the indolepropionic acid experiments. Inasmuch as all animals receiving this product gave entirely uniform results, it has seemed unnecessary to present the growth curves of more than two litters. Chart I shows the effects of indolepropionic acid when incorporated in the basal diet; Chart II, the influence of the compound when administered in the vitamin pills. As will be observed, the growth curves are practically parallel to those of the rats on the non-supplemented ration. Evidently, indolepropionic acid, whether fed as a component of the ration or separately, is wholly incapable of supporting the growth of rats upon a tryptophane-deficient basal diet. This finding is in accord not only with the experiments of Jackson (1928), and of Heft and Sherwin (1926) referred to above, but also with the fact that imidazole propionic acid (Cox and Rose, 1926), and β -dithiodipropionic acid (Westerman and Rose, 1927) are incapable of replacing histidine and cystine, respectively.

The results of the experiments with indolepyruvic acid are summarized in Chart III. During the fore period, slow growth occurred in all cases because of the tryptophane introduced in the yeast. Somewhat more rapid but distinctly subnormal growth was manifested by the rats which received an additional 0.02 per cent of tryptophane in the basal ration. *But when indolepyruvic acid was either incorporated in the basal ration at a 0.2 per cent level, or fed separately in 10 mg. doses at 12 hour intervals, the rats responded with a pronounced increase in growth rate which continued for the entire 28 day period.* Meanwhile, the control rat without the synthetic product made a less satisfactory gain than during the fore period. Furthermore, when the indolepyruvic acid was withdrawn from the food all of the rats declined. *The results clearly demonstrate that indolepyruvic acid is capable of serving in place of tryptophane for purposes of growth.* It should be pointed out also that the inclusion in the food of tryptophane instead of indolepyruvic acid during the last 28 days of the experiments induced increases in weight which are quite comparable to, or in some cases less satisfactory than, those which resulted from the

CHART III
INDOLE PYRUVIC ACID

- A - Basal Diet unsupplemented
B - Basal Diet containing 0.2% Tryptophane
C - 0.2% Indole Pyruvic Acid in Basal Diets
D - 10 mg. of Indole Pyruvic Acid fed separately at 12 hour intervals
E - 0.4% Indole Pyruvic Acid in Basal Diet B
F - 0.2% Tryptophane in Basal Diets
G - 10 mg. of Tryptophane fed separately at 12 hour intervals

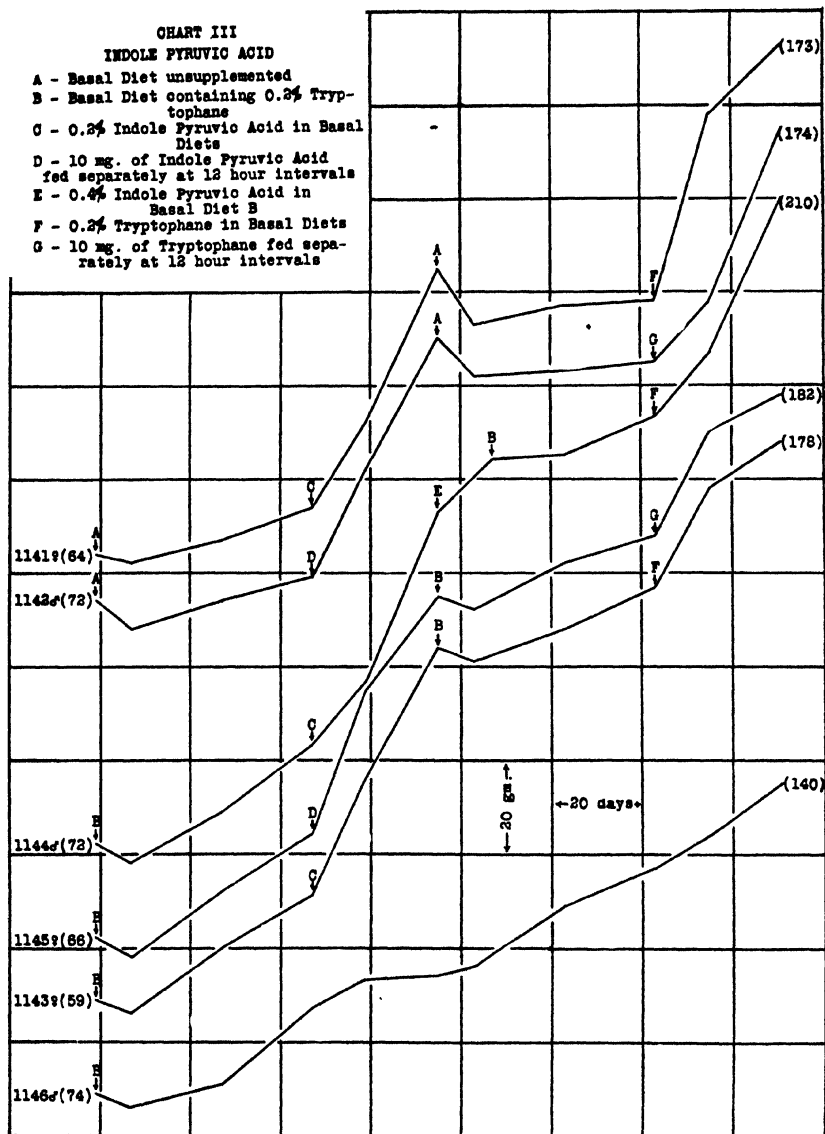


CHART III.

TABLE I.

Comparative Growth of Animals upon Diets Supplemented with 3-Indolepyruvic Acid and with Tryptophane.

Rat No. and sex.	Total gain during 28 days with indolepyruvic acid.	Total gain during 28 days with tryptophane.	Basal diet.
	gm.	gm.	
1141 ♀	51	55	Without added tryptophane.
1142 ♂	51	51	
1143 ♀	53	31	With 0.02 per cent tryptophane.
1144 ♂	50	47	
1145 ♀	51	30	

TABLE II.

Growth upon a Tryptophane-Deficient Basal Diet Supplemented with Indolepropionic Acid in the Food Mixture.

Rat No. and sex.	Average daily food consumption.	Average daily change in weight.	Supplement.
	gm.	gm.	
831 ♂	4.5	-0.23	0.37 per cent indolepropionic acid.
832 ♀	4.1	-0.25	0.37 " " " "
833 ♀	4.3	-0.23	0.37 " " " "
834 ♂*	9.2	+1.55	0.2 " " tryptophane.
835 ♂*	8.4	+1.86	0.2 " " " "
836 ♂	5.2	-0.25	None.
837 ♀	4.7	-0.24	"

* Part of the growth curves of Rats 834 and 835 could not be included on Chart I. The final weights of the animals at the end of 80 days were 222 gm. and 235 gm., respectively.

TABLE III.

Growth upon a Tryptophane-Deficient Basal Diet Supplemented with Indolepropionic Acid Fed Separately at 12 Hour Intervals.

Rat No. and sex.	Average daily food consumption.	Average daily change in weight.	12 hr. supplement.
	gm.	gm.	
879 ♀	5.1	-0.23	None.
880 ♀	4.7	-0.45	18.5 mg. indolepropionic acid.
881 ♀	5.0	-0.39	18.5 " " "
882 ♀	8.6	+0.95	10 " tryptophane.

TABLE IV.

Growth upon a Tryptophane-Deficient Basal Diet Supplemented with Indolepyruvic Acid.*

Rat No. and sex.	Days.	Average daily food con- sump- tion.	Average daily change in weight.	Supplement.
		gm.	gm.	
1141 ♀	1-48	3.7	+0.21	None.
	49-76	6.4	+1.82	0.2 per cent indolepyruvic acid in diet.
	77-124	5.4	-0.15	None.
	125-152	7.1	+1.96	0.2 per cent tryptophane in diet.
1142 ♂	1-48	3.4	+0.10	None.
	49-76	5.1	+1.82	10 mg. indolepyruvic acid at 12 hour intervals.
	77-124	5.2	-0.10	None.
	125-152	6.1	+1.82	10 mg. tryptophane at 12 hour intervals.
1143 ♀	1-48	4.3	+0.46	None.
	49-76	7.3	+1.89	0.2 per cent indolepyruvic acid in diet.
	77-124	7.9	+0.27	None.
	125-152	7.6	+1.11	0.2 per cent tryptophane in diet.
1144 ♂	1-48	4.3	+0.44	None.
	49-76	6.4	+1.79	0.2 per cent indolepyruvic acid in diet.
	77-88	5.9	+0.92	0.4 " " " " " "
	89-124	7.5	+0.25	None.
	125-152	7.4	+1.68	0.2 per cent tryptophane in diet.
1145 ♀	1-48	4.4	+0.46	None.
	49-76	7.1	+1.82	10 mg. indolepyruvic acid at 12 hour intervals.
	77-124	7.5	+0.27	None.
	125-152	7.4	+1.07	10 mg. tryptophane at 12 hour intervals.
1146 ♂	1-48	4.3	+0.38	None.
	49-76	4.1	+0.25	"
	77-124	6.0	+0.48	"
	125-152	6.5	+0.64	"

* Rats 1141 and 1142 received no tryptophane in the basal ration. Rats 1143 to 1146 inclusive received a basal diet which contained 0.02 per cent tryptophane.

administration of the synthetic compound. The total gains under the two dietary conditions are summarized in Table I. Naturally, such comparisons should not be interpreted too literally, inasmuch as the rats were older when they received tryptophane than when provided with the synthetic material. But despite the obvious difference in the age of the animals when the synthetic and natural products were administered, the data appear to warrant the belief that the indolepyruvic acid underwent almost a quantitative asymmetric transformation into the amino acid. In any event, the compound certainly manifests a remarkable efficiency in replacing tryptophane.

In Tables II to IV are shown the average daily food consumption and average daily change in body weight of each rat. As pointed out in the preceding paper, regardless of how the supplementing agents were administered, whether in the basal diet or in the vitamin pills, if the food was thereby rendered satisfactory for growth a stimulation in appetite resulted. On the other hand, when the supplementing agent (indolepropionic acid) was incapable of improving the quality of the ration, no increase in food intake occurred.

The above experiments furnish proof of the replacement of a second "indispensable" amino acid by a synthetic non-amino compound. It is interesting to note that both tryptophane and histidine are replaceable by the corresponding α -ketonic acids (*cf.* Harrow and Sherwin, 1926) and that histidine is also replaceable by the corresponding α -hydroxy acid (*cf.* Cox and Rose, 1926). On the other hand, appropriate α -hydroxy acids appear to be incapable of serving in place of lysine (McGinty, Lewis, and Marvel, 1924-25), tryptophane (Jackson, 1927), or cystine (Westerman and Rose, 1928).

SUMMARY.

3-Indolepropionic acid and 3-indolepyruvic acid have been fed to rats upon tryptophane-deficient diets in order to determine whether the substances in question are capable of serving in place of the amino acid for purposes of growth. The results indicate that 3-indolepropionic acid is entirely unable to meet the requirements of the organism in place of tryptophane.

On the other hand, the addition to the tryptophane-deficient

diet of 3-indolepyruvic acid leads to an immediate resumption of growth at a rate quite comparable to that induced by the equivalent quantity of tryptophane. It is evident that under the conditions of the experiments the synthetic product substitutes for tryptophane, probably by being transformed into the amino acid.

It was formerly shown in this laboratory that 4-imidazole lactic acid induces the growth of rats upon a histidine-deficient ration. The present experiments provide proof of the replacement of a second "indispensable" amino acid by a synthetic compound.

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THE ACID-BASE EQUILIBRIUM OF THE BLOOD IN ECLAMPSIA.

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The most outstanding chemical findings, so far reported, in patients suffering from eclampsia are the decreased CO_2 -combining power and the increased uric and lactic acids in the blood. The CO_2 -combining power is often in the neighborhood of 20 volumes per cent, and it is not unusual to observe uric acid values above 10 mg. and lactic acid above 100 mg. per 100 cc. of blood (1-3). The hydrogen ion concentration of the blood has been determined in a few isolated cases of eclampsia (4, 5), but there has been no complete study of the acid-base equilibrium in this disease.

It seemed to us that the markedly lowered CO_2 -combining power, often observed in the eclamptic patient, may be evidence of a true acidosis. In this connection, one is sometimes impressed with the exceedingly short duration of the disease, ending in death. In this clinic, we have observed eclamptic patients dying 2 to 3 hours after the first convulsion; furthermore, all such patients had exceedingly low CO_2 -combining power. These deaths may be due to a rapidly developing acidosis, and it is because of this probability that we undertook to make a complete study of the acid-base equilibrium in eclampsia.

Collection of Blood.

All specimens of blood, with the exception of those of eclamptic patients, were obtained in the morning before breakfast. Each blood sample was secured in two fractions, the first being drawn under oil and the second into 50 cc. centrifuge tubes. The former was employed for the determinations of hydrogen ion concentra-

tion and CO_2 content. This particular fraction of the blood was always taken without the use of a tourniquet, and with the patient resting in bed. The greatest possible care was exercised to prevent CO_2 loss, and to this end the following procedure was employed: The blood is drawn from the patient's median basilic vein into a 30 cc. syringe, into which have previously been introduced 5 cc. of sterile light oil, of tested neutrality, the bore of the needle being also completely filled with this fluid. The blood is immediately transferred under oil to two 15 cc. centrifuge tubes in such a manner that the tubes retain only a thin layer of supernatant oil. A vaccine stopper, through which a thin bore needle has been introduced, is now inserted into the centrifuge tubes. As this stopper is pushed downwards, the thin layer of oil and a few drops of blood escape through the needle, which is now immediately withdrawn and the blood in this way effectively protected from the air. The blood is then centrifuged, and as it is no longer in contact with oil, the error due to escape of CO_2 from blood to oil is obviated. The centrifugation is carried out at 1000 R.P.M. for 10 minutes. The blood is again covered with oil by means of introducing through the vaccine stopper a needle attached to a small syringe, both needle and syringe being filled with oil. It is not necessary to force the oil downwards, as it is drawn into the tube by the vacuum produced by raising the stopper out of the tube. The serum can now readily be drawn off for hydrogen ion and CO_2 determinations.

The second fraction is taken with minimum stasis, allowed to clot in the 50 cc. centrifuge tubes, and centrifuged at 1000 R.P.M. for 10 minutes. The serum from this fraction is used for all other determinations.

Methods.

The *pH* was measured electrometrically, with a Michaelis U electrode (6) and a Leeds and Northrup potentiometer type K. Triplicate determinations were made on each blood sample with a precision of 0.01 pH.

The *carbon dioxide* content was determined by the Van Slyke and Neill method (7) in a calibrated constant volume Van Slyke pipette.

Total Base.—Serum was ashed by the method of Van Slyke,

Hiller, and Berthelsen (8), and benzidine precipitation and titration were carried out in accordance with the procedure of Stadie and Ross (9).

Chlorides were determined by the Whitehorn method (10).

Proteins were estimated by macro Kjeldahl determination on 1 cc. specimens, determined non-protein nitrogen being deducted.

Non-protein nitrogen was determined by the Folin-Wu method (11).

Sulfates were determined by the Denis and Reed nephelometric method (12).

Acetone bodies were estimated by the method of Van Slyke and Fitz (13).

Organic acids in blood were determined by the method of Perlzweig and Delrue (14).

Organic acids in urine were determined by the Van Slyke and Palmer method (15).

Calculations.

In order to express all values in millimolar equivalents, the following formulæ were employed:

$$\text{BHCO}_3 = \frac{\text{CO}_2 - \frac{\text{CO}_2}{\text{antilog}(\text{pH} - \text{pK}) + 1}}{22.4} \times 10 \text{ (Peters, et al. (16)).}$$

$$\text{BP} = 0.104 \times \text{gm. protein per 100 cc.} \times (\text{pH} - 5.08) \times 10 \text{ (Van Slyke, et al. (17)).}$$

$$\text{BCl} = \frac{\text{mg NaCl per 100 cc.}}{58.46} \times 10$$

$$\text{BPO}_4 = \text{mg. P per 100 cc.} \times 0.58 \text{ (Henderson (18)).}$$

$$\text{BSO}_4 = \text{ " S " 100 " } \times 0.6 \text{ (Peters, et al. (16)).}$$

Results.

In Table I are given our results for normal non-pregnant women. In this group of cases we did not determine the blood organic acids, and these are, therefore, represented in the column "undetermined acid." Table II gives the findings in normal pregnancy. In Table III, which presents the results in eclampsia, are given the

values found during the period of convulsions and while the patients were severely ill. A more detailed report covering the changes throughout the disease and during convalescence will appear in a later publication.

From a comparison of the values expressed in Tables I and II, it is manifest that normal pregnancy is accompanied by a reduction in fixed base, as well as a decrease in the serum anions, protein, and bicarbonate. The reduction in total base of the serum in normal pregnancy averages 8 mm. Gestation is accompanied by a normal hydrogen ion concentration, averaging pH 7.37.

The findings in eclampsia, as shown in Table III, are very striking when compared with the normal pregnancy values. The

TABLE I.
Acid-Base Equilibrium in Normal Non-Pregnant Women.

Case No.	Non-protein nitrogen.	Proteins.	BP	CO ₂	BHCO ₃	NaCl	BCl	Phosphorus.	BPO ₄	Sulfur.	BSO ₄	Total acid.	Total base.	Undetermined acid.	pH
	mg. per 100 cc.	per cent	mm	vol. per cent	mm	mg. per 100 cc.	mm	mg. per 100 cc.	mm	mg. per 100 cc.	mm	mm	mm	mm	
24410	28.9	6.7	16.0	60.7	25.8	609	104.0	2.2	1.3	0.8	0.5	147.6	154.7	7.1	7.37
24239	27.7	6.9	15.9	58.2	24.3	627	107.2	2.3	1.3	1.0	0.6	149.3	151.9	2.6	7.31
23187	20.7	7.3	17.2	61.6	26.0	636	108.7	2.1	1.3	0.7	0.4	153.6	159.4	5.8	7.33
Average.	25.8	7.0	16.4	60.2	25.4	624	106.6	2.2	1.3	0.8	0.5	150.2	155.3	5.2	7.34

total base amounts to about 3 mm more than in normal pregnancy, but is below the normal non-pregnant limits. Nevertheless, there is a definite reduction in BHCO₃, which is more than counter-balanced by the marked increase in organic acids. The most outstanding changes noted in Table III are the very low pH readings, the definitely increased organic acids, and the reduction in BHCO₃.

The millimolecular BHCO₃ concentration and pH values for our normal non-pregnant, pregnant, and eclamptic women are plotted in Chart I, in which we have employed the graph devised by Van Slyke (19). It will be noted that our normal non-pregnant values all fall within Area 5, which represents a normal acid-base balance. Normal pregnancy values fall close to the boundary separating

TABLE II.
Acid-Base Equilibrium in Normal Pregnancy.

Case No.	Non-protein nitrogen.	Proteins.	BP	CO ₂	BHCO ₃	NaCl	BCl	Phosphorus.	BPO ₄	Organic acids.	Sulfur.	BSO ₄	Total acid.	Total base.	pH	Duration of pregnancy.
	mg. per 100 cc.	per cent	mM	vol. per cent	mM	mg. per 100 cc.	mM	mg. per 100 cc.	mM	mM	mg. per 100 cc.	mM	mM	mM		
24038	23.0	6.4	15.2	48.1	20.4	620	106.0	2.1	1.2	1.7*	0.7	0	4144.9	144.9	7.38	2 days ante partum.
24547	20.0	6.1	13.4	47.2	20.1	596	102.0	1.9	1.1	8.2	0.9	0.5	145.9	143.8	7.41	5½ mos.
T-41929	31.6	5.8	13.6	51.2	21.6	610	104.3	1.8	1.0	6.8	0.7	0	4147.5	152.5	7.33	At term.
Average.	24.9	6.1	14.1	48.8	20.7	608	104.1	1.9	1.1	5.6	0.8	0.5	146.1	147.0	7.37	

Estimated.

TABLE III.
Acid-Base Equilibrium in Eclampsia.

Case No.	Non-protein nitrogen.	Proteins.	BP	CO ₂	BHCO ₃	NaCl	BCl	Phosphorus.	BPO ₄	Organic acids.	Sulfur.	BSO ₄	Total acid.	Total base.	pH	Time specimen taken.
	mg. per 100 cc.	per cent	mm	vol. per cent	mm	mg. per 100 cc.	mm	mg. per 100 cc.	mm	mm	mg. per 100 cc.	mm	mm	mm		
23309	61.8	5.3	10 2	29.1	11 5	631	107 8	1 9	1.1	18 0	1.5	0 9	149.4	151 3	6 99	In coma. 45 sec. after 4th con- vulsion.
23925	33.3	6.8	14 3	35.6	14 4	621	106 2	1.9	1 1	14 5	0 9	0 5	151 3	154 6	7 07	In coma. 30 sec. after 4th con- vulsion.
25222	25.0	6.4	13.4	42.7	17 5	597	102 0	1 8	1 0	20 5			155 0	152 8	7 13	In coma. About 30 min. after 4th convulsion.
S-81929	32.1	6 9	13 6	40 2	15.9	596	101.9	2 8	1.7	13.7			147.5	153 4	6 98	In coma. 5 min. after 10th con- vulsion.
Average.	38.0	6.4	12 9	36 9	14 8	611	104 5	2 1	1 2	16.7	1 2	0 7	150 8	153.0	7.04	

Areas 5 and 6. In other words, there is a tendency in normal pregnancy towards the compensated alkali or CO_2 deficit area. This is due to the lowered BHCO_3 concentration.

All our eclamptic cases fall in Area 9, which represents an uncompensated alkali deficit. This true acidosis, which we have found in all our cases of severe eclampsia, seems to be due to the

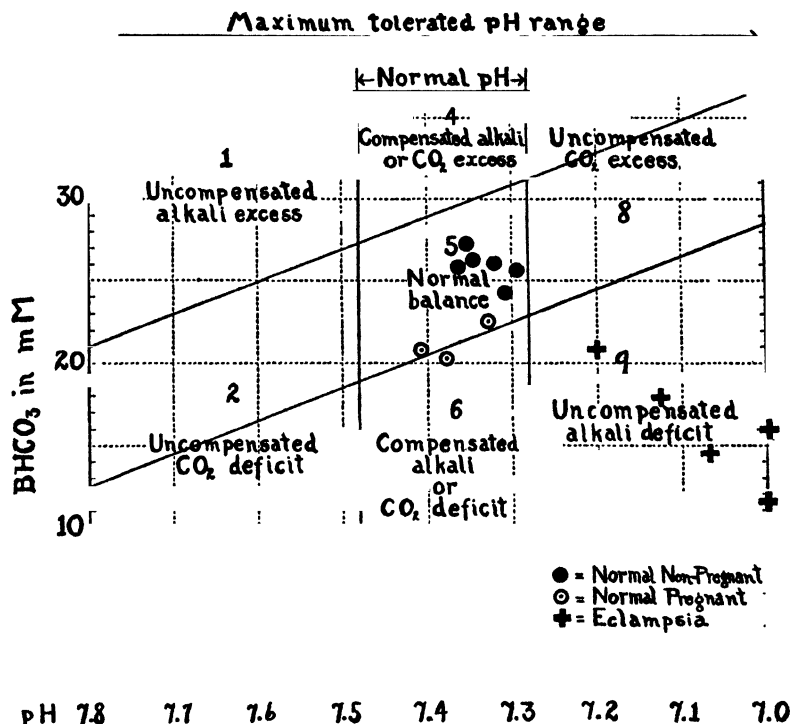


CHART I. Variations of the BHCO_3 and pH in serum of normal non-pregnant, normal pregnant, and eclamptic women.

marked accumulation of organic acids (probably chiefly lactic) in the blood, and to a concomitant reduction in BHCO_3 . We may state that so far we have found normal values for blood acetone bodies in eclampsia, and ketosis therefore probably does not play a rôle in the production of this acidosis. In this connection it is interesting to note that the organic acids in the urine in eclampsia

are usually markedly increased. The following are our findings for the patients represented in Tables I to III.

No. of cases.	<i>Organic Acids in Urine.</i>	
	Type.	Average 0.1 N organic acid. cc. per 24 hrs.
3	Normal non-pregnant.	338
2	" pregnant.	775
3	Eclampsia, severe.	1168

DISCUSSION.

Normal Pregnancy.—Oard and Peters have recently given an excellent review of the literature on serum electrolytes in normal pregnancy (20). These authors made a very complete study of the concentration of acid and base in the serum of normal pregnancy, and showed that the total base in pregnancy is lowered about 8 mm from the normal, and that there is also a concomitant and equal reduction of the anion content of the serum in pregnancy. This reduction they found to be in serum protein, serum bicarbonate, and organic acid. Our own results are in full agreement with those of Oard and Peters, except that we were unable to note a reduction in organic acids. It should be noted, however, that Oard and Peters calculated the organic acids as "undetermined acid," while we measured it electrometrically by the method of Perlzweig. It should also be stated that while this method gave very good results when we were dealing with fairly large quantities of organic acids, as in eclampsia, our results were not so satisfactory when the amounts to be determined were less than 8 mm of acid. This may perhaps explain the slight discrepancy between the results of Oard and Peters and our own figures.

Our pH determinations show that normal pregnancy is not accompanied by any change from the normal non-pregnant value. We have been unable to find in the literature electrometric pH determinations in pregnancy with which to compare our results.

The term "acidosis of pregnancy," as pointed out by Oard and Peters, is misleading, as normal pregnancy is not associated with an increase of abnormal acids, but rather with a reduction of alkali reserve and a decrease in total base. We agree with these authors that the reduction in fixed base in the serum in pregnancy cannot readily be explained on the basis of fetal requirements,

acid production and excretion, or hyperventilation. At present, we are unable to give a satisfactory explanation for this serum base decrease which accompanies normal pregnancy.

Eclampsia.—The literature contains no complete study of the acid-base equilibrium in the serum of eclamptic women. It is well known, however, that the CO_2 -combining power of the blood is often markedly lowered in severely ill eclamptic patients. Furthermore, Zweifel (21), Bokelmann (22), Kienlin (23), Loeser (24), Schultze (25), and Stander and Radelet (3), demonstrated an increase in lactic acid in the blood of eclamptic women. Hasselbalch and Gammeltoft examined four eclamptic patients and noted in two cases an increase in the fixed acidity of the blood, while in the other two cases, the pH of the blood was normal. From these considerations, one may suspect that the coma of eclampsia is associated with a true acidosis. Our total electrolyte studies on the sera of five eclamptic patients fully confirmed this hypothesis. All our patients, while in coma, showed an acid-base balance falling in Van Slyke's Area 9, which represents an acidosis resulting from an uncompensated alkali deficit. The alkali reserve has been lowered below the extreme normal limit.

The total base in eclampsia is not lower than in normal pregnancy—on the contrary, it is slightly higher (3 mm). A previous study (26) on the cations of the blood in eclampsia gave the following averages, as compared with normal pregnancy.

Normal pregnancy.				Eclampsia.			
Na	=	325	mg. or 141.3 mm	332	mg. or 144.3 mm		
K	=	21.2	" " 5.4 "	21.3	" " 5.4 "		
Ca	=	9.6	" " 4.8 "	10.7	" " 5.4 "		
Mg	=	2.3	" " 1.9 "	2.8	" " 2.3 "		
Total	=		153.4 "				157.4 "

These figures agree very satisfactorily with our determinations on total base, as eclampsia gave a value 3.2 mm more than normal pregnancy, and from the above calculations based on our previous study, eclampsia shows 4.0 mm more than normal pregnancy.

Certain anion concentrations are markedly altered in eclampsia. BHCO_3 and serum protein are reduced, while organic acids are markedly increased. We have not determined the nature of these organic acids, although our evidence so far indicates that they are not ketones, but probably mainly lactic acid. The outstanding

changes on the acid side are the accumulation of organic acid and the decrease in BHCO_3 . Chlorides, phosphates, and sulfates are within normal limits. The pH of the eclamptic blood is at the very lower limit of the "maximum tolerated pH range," being 7.04.

From these figures it is evident that the uncompensated acidosis in eclampsia is so marked that it in itself may cause the death of the patient. Whether the eclamptic convulsions cause this low pH, or are the result of it, cannot be determined until more work has been done on the etiology of this acidosis.

Our electrolyte studies on eclamptic sera lead us to the main conclusion that many patients undoubtedly succumb to this disease because of the marked acidosis due to an uncompensated alkali deficit, and that a certain number of these may be saved by the rigorous use of antiacidosis treatment, such as bicarbonate.

CONCLUSIONS.

1. Normal pregnancy is associated with a reduction in total serum base, amounting to about 8 mm.

2. This reduction in total base is accompanied by a decrease in serum protein and serum bicarbonate. The other anion concentrations are within the normal non-pregnant limits.

3. There is no increase in organic acids, nor an accumulation of abnormal acids during normal pregnancy.

4. The pH of the blood in uncomplicated gestation is within the normal range.

5. Severe eclampsia is associated with a true acidosis, due to an uncompensated alkali deficit.

6. A marked increase in organic acids in the blood accompanies eclampsia.

7. The pH of the blood in severe eclampsia is decidedly lowered, averaging about 7.04.

8. The marked BHCO_3 decrease, with its accompanying acidosis, observed in eclampsia, deserves special attention in the treatment of the disease.

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THE PRODUCTION OF ORGANIC ACIDS BY EXCISED INTESTINES.

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In a study of the buffering of physiological saline solutions, it was found that excised intestines when suspended in a warm bath of Locke's solution, containing 0.1 per cent of dextrose, produce appreciable quantities of fixed organic acids. Evans (1) describes the appearance of lactic acid during the contraction of the smooth muscle under anaerobic conditions, presumably from glycogen. More recently, Ronzoni (2) working with the chicken gizzard found that lactic acid is formed from glucose-containing Ringer's solution, which does not occur when glucose is replaced by glycogen. Rona and Neukirch (3) have shown that the activity of the cardiac muscle is connected with the consumption of dextrose; and the formation of lactic acid during the contraction was later established by Redfield and Medearis (4) and others. However, as regards the acid formation in excised intestines, it appeared to us unsafe to take the explanation of muscular contraction for granted, in view of the possible effects of the digestive enzymes, glandular cells, and fermentative bacteria. The following investigation was undertaken to differentiate these factors. In these experiments the pH was checked with the colorimetric method, with the standard buffers of Palitzsch (5) and McIlvaine (6) and phenol red as indicators. The organic acid was determined by the method of Van Slyke and Palmer (7), which is based on the fact that relatively little strong mineral acid is necessary to change the pH of aqueous solutions from pH 8.0 to 2.69 if the only electrolytes present are the alkali salts of strong mineral acids, such as sulfates and chlorides. If, however, the salts of weaker acids are present, the addition of nearly a molecular ratio of HCl is required in order to cause the above change of the pH. Therefore, the phosphates and carbonate are first re-

moved by shaking with $\text{Ca}(\text{OH})_2$, and the filtrate is titrated with 0.2 N HCl against phenolphthalein until the pink color just disappears (pH 8.0), and then the titration is continued against tropeolin OO until the color matches with a standard. From the difference between these two titrations the amount of cc. of 0.1 N organic acid per liter is calculated.

The experiments were carried out with pieces of excised rabbit ileum, a meter long, by the method described by von Oettingen, Sollmann, and Ishikawa (8) with Locke's solution, containing NaCl 0.9, KCl 0.042, $\text{CaCl} \cdot 2\text{H}_2\text{O}$ 0.024, NaHCO_3 0.008, and dextrose 0.1 gm. per 100 cc. The intestine was suspended in

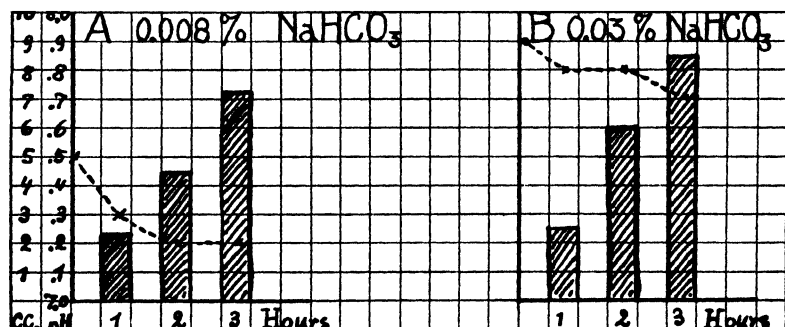


CHART I. Acid formation by perfused rabbit ileum with different concentrations of NaHCO_3 . The dotted curve shows the changes of the pH, the vertical columns the amount of fixed organic acid in cc. of 0.1 N organic acid per liter.

5 liters of this solution and also perfused through the lumen; the temperature of the bath was 38° , and the aeration unless stated otherwise was about 100 cc. of air per minute. The pH and the organic acid were determined at intervals of 30 or 60 minutes for 3 hours. Under these conditions there is no formation of fixed organic acids in the Locke's solution in the absence of intestine.

Chart I, A gives the average of two experiments with the intestine in the Locke's solution, as described. It shows that the pH falls 0.3 point, from 7.5 to 7.2 within 3 hours, and that the total amount of organic acid formed during this time is 2.2, 4.5, and 7.2 cc. of 0.1 N organic acid per liter, after each hour. In the next series (Chart I, B), the alkalinity of the buffer solution was

increased by using 0.03 per cent instead of 0.008 per cent sodium bicarbonate. The results obtained are essentially the same as in the former series. In another series of experiments, the intestine was not perfused, in order to suppress peristalsis, and thus to find out to what extent the acid formation depends on the peristaltic activity. The acid formation was essentially the same as in the preceding groups, so that the acid formation is largely, if not entirely, independent of the peristaltic activity of the intestine.

The relation of the acid formation to the amount of tissue was next studied. With only one-third as much intestine as in the preceding experiments, the acid increased only from 0 to 4 cc. of 0.1 N organic acid per liter within 3 hours; so that the acid formation is evidently proportional to the quantity of tissue. It was also found that the acid formation continues only as long as the intestine is left in the solution, for if the intestine was removed at the end of the 1st hour, further acid formation was practically absent.

In order to find out which tissue of the intestines plays the important part in the formation of fixed organic acids, the effect of the three constituents of the intestinal wall was studied separately. For this purpose, the mucosa, serosa, and muscularis of beef intestine were used, and since it was impossible to get the muscularis absolutely free from serosa and mucosa material, one series of experiments was also performed with muscular tissue from beef uterus. In these experiments, 3.5 gm. of the minced tissue were suspended in 1 liter of Locke's solution and kept under conditions analogous to those of the preceding experiments. With mucosa, 11.5 cc. of 0.1 N organic acid per liter were produced in the solution within 3 hours; the same amount of serosa under the same conditions yielded only 4.5 cc. and of muscularis, 6.3 cc. of 0.1 N organic acid per liter (average of six experiments in each group). For the reason stated above, the experiments were repeated with equal amounts of minced beef uterus, but no organic acid was formed under these conditions (three experiments). It appears therefore that the mucosa is chiefly involved in the formation of fixed organic acids, and that serosa and muscularis have comparatively little or no effect. It was also confirmed that boiling the mucosa suspension, for 10 minutes, prevents the formation of acid.

The chemical conditions under which the acid formation occurs in the presence of the intestine were then studied; namely, the importance of the presence of dextrose, the presence or absence of oxygen, and the effect of changes of the hydrogen ion concentration. For this purpose, long pieces of rabbit ileum were used as in the first series, the conditions being varied as indicated.

It was found that acid formation does not occur if dextrose is absent from the Locke's solution, and, therefore, it appears that the acid is derived from the dextrose of the bath and not from any tissue material. Further, it was found that the acid formation is independent of the presence of oxygen, since equal amounts of fixed organic acid are formed when the bath is aerated with air, with hydrogen, and without any aeration. Therefore, the acid formation is probably an anaerobic process. This indicates that the acid formation does not take place within the living cells of the intestinal tissue, either glandular or muscular.

In the next series of experiments, the influence of the pH of the medium on the acid formation was studied, since enzymatic processes are generally greatly influenced by the hydrogen ion concentration. For these experiments, beef mucosa in the same quantities as in previous experiments was used, and the pH of the Locke's solution was adjusted to different levels by means of varying amounts of NaHCO_3 and HCl . It was found that under the different conditions the acid formation is practically the same whether the initial pH was 7.9, 7.0, 6.7, 5.0, or 3.0, so that the acid formation is largely independent of the pH, at least on the acid side, and, therefore, is probably not due to the action of extracellular enzymes, but rather to some intracellular process.

It remained to determine whether tissue cells or bacteria are the cause of this phenomenon. It is well known that certain bacteria as *Bacillus subtilis*, *Bacillus prodigiosus*, *Streptococcus lactis*, *Lactobacillus leichmanni*, and *Bacillus coli* are able to produce lactic acid. Takao (9) has shown this for glucosamines and Kinura (10) for dextrose. It was therefore possible that we were dealing with the action of bacteria living in the intestinal tract. Dr. E. E. Ecker kindly made for us cultures from the ileum of rabbits, and it was found that a loop from these cultures suspended in 30 cc. of dextrose Locke's solution produced a very sharp fall of the pH from 7.5 to 6.5 within 3 hours, with the production of variable but large amounts of fixed organic acid. There was thus

a definite possibility that the acid formation is due to the fermentation of the dextrose by the intestinal bacteria. The following two methods were used to decide more definitely between such bacterial fermentation and glandular activity.

A meter of split intestine was suspended in 5 liters of warmed and aerated dextrose Locke's solution for an hour, so that the interior of the intestine was exposed to the solution. After 1 hour, the acid formation was measured and found about twice as high as with perfused intestine, as would be expected in view of the higher activity of the exposed mucous layer. The piece of intestine was then removed and the heating continued for 2 hours longer and the acid again measured. No increase would be expected if the acid formation were due to the digestive cells, since these would be removed from the system. On the other hand, bacteria would have been diffused through the solution, so that removal of the intestine would not arrest the progress of the acid formation. In three experiments, it was found that the acid formation continued, to 16 cc. of 0.1 N organic acid per liter. This indicates that bacteria play a major part, at least, in the acid formation.

The second method of distinction was based on the fact that the bacteria of the small intestines of rabbits are confined largely to the lower levels. Therefore, if the acid formation is bacterial, materially less organic acid should be formed when the perfused duodenum and jejunum are used than when the ileum is included, while the acid formation would be reduced only proportionally to the quantity of the tissue, if the intestinal cells played the major rôle. Three experiments showed uniformly that no organic acid is formed when only the upper portions of the intestine are perfused. This shows clearly that the intestinal tissue as such plays no significant part in the formation of organic acid from the sugar, and that this is due to the activity of the bacteria which flourish in the lumen of the ileum.

SUMMARY AND CONCLUSION.

Organic acids are formed from dextrose when rabbit ileum is suspended in warmed dextrose containing Locke's solution. This is independent of muscular activity of the intestine. It is effected mainly by the mucosa. The serosa or muscularis has little or no action.

The process is anaerobic, occurring equally well in an atmosphere of hydrogen or without aeration.

The acid-forming agent is destroyed by boiling, but is not affected by changes of the pH between 7.9 and 3.0. The acid formation is therefore probably not due to an extracellular enzyme. The anaerobic character of the process indicates that it is due to the living tissue, and that it must be ascribed either to endocellular tissue ferments or to bacteria. Acid formation is produced by bacteria cultured from the ileum.

With the split intestine, the acid formation is twice as high in the first 2 hours as with the perfused intestine. After the removal of the split intestine, the acid formation still progresses rapidly.

No acid formation is produced when the relatively sterile duodenum and jejunum are perfused instead of the ileum.

The formation of fixed organic acids, which occurs when small intestines are suspended in Locke's solution is due to the fermentation of the dextrose by the bacteria that flourish in the lumen of the ileum and not to muscular or enzymatic activity of the intestinal cells proper.

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THE DETERMINATION OF ISOPROPYL ALCOHOL IN THE PRESENCE OF ACETONE IN THE URINE.*

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Isopropyl has replaced ethyl alcohol in many such pharmaceutical preparations and cosmetics as mouth washes, dental solutions, and hair tonics. Although the physiology and toxicity of this material are fairly well known, a thorough knowledge of the metabolism involves a method for the separate determination of isopropyl alcohol and acetone, the chief product of its oxidation in the body. Many of the useful procedures for detecting acetone will also give positive results with the alcohol, either as such, or after decomposition under the conditions of the reaction. The present study describes a procedure for the determination of acetone and isopropyl alcohol when both are present in the urine. Under the conditions of the method isopropyl alcohol gives a maximal yield of acetone but it is realized that other compounds containing the isopropyl group would yield some acetone.

The principle of the method consists in the oxidation of the isopropyl alcohol to acetone and the measurement of the resulting acetone as the mercuric sulfate complex of Denigès (1898). The preformed acetone is determined on a separate sample by distilling into hydroxylamine hydrochloride and titrating the acid liberated in the condensation of the acetone with the hydroxylamine hydrochloride. The procedure for the total acetone is essentially like that of Van Slyke (1917) for acetone bodies in the urine.

Determination of Total Acetone.

The isopropyl alcohol is oxidized by refluxing with dichromate and sulfuric acid. The resulting acetone together with the pre-

* The data in this paper are taken from an essay presented by Charles A. Cook for the degree of Master of Science, Yale University, 1930.

formed acetone forms a complex mercury salt in the presence of mercuric sulfate and sulfuric acid (Denigès' reagent). This crystalline compound is filtered off, dried, and weighed. It is approximately 20 times heavier than the acetone it represents (Denigès, 1898; Oppenheimer, 1899; Sammett, 1913) and this factor is used to translate the value of the mercury complex to that of acetone. The sulfuric acid solution of mercuric sulfate at the temperature maintained oxidizes most of the alcohol to acetone. It is thus impossible to differentiate between acetone and alcohol values by this procedure and preformed acetone must be determined by a different method on a separate sample.

Removal of Interfering Substances.—It is necessary to remove extraneous substances which might also yield a precipitate with Denigès' reagent. The same procedure as that employed by Van Slyke (1917) is used. It consists of precipitating the glucose as the copper complex (Salkowski, 1879) which also carries down with it other interfering substances.

Effect of Length of Time of Heating.—A series of determinations was made to obtain the optimum and most practical duration of time for the oxidation and precipitation reaction. The concentration of the reagents was maintained constant and the time of heating varied. A reaction time of 1 hour gave the maximum return of acetone. The theoretical return can be approached in two ways: a long period of reaction with a low concentration of reagents, or a short period with greater concentrations of oxidizing agents.

Effect of Variation of Dichromate Concentration.—The concentration of dichromate alone was increased in steps and the effect on acetone recovery noted. The acid dichromate mixture has a tendency to split hydroxy compounds and gives rise to acids and ketones containing a smaller number of carbon atoms. With progressively increasing concentration a rise to a maximum is obtained, followed by a decline. This decline is undoubtedly due to rupture of the acetone molecule on strong oxidation and the formation of lower aliphatic compounds which do not give a precipitate with Denigès' reagent. For practical reasons 40 cc. of dichromate and a period of 1 hour's heating were adopted as standard procedure although 30 cc. of dichromate for 90 minutes also gave acceptable returns.

Effect of Variation of Sulfuric Acid Concentration.—By varying the ratio of acid concentration to that of the dichromate it was found that the reaction could be altered from one of mild oxidation to one of powerful decomposition. The acid plays such a profound rôle that it was kept at a minimum and the dichromate altered to secure the most favorable reaction. It is well to remember that an adequate concentration of acid must be maintained to prevent precipitation of basic mercuric sulfate. The concentration of the mercuric sulfate cannot be increased greatly without adding more acid for the same reason.

Effect of Alcohol Concentration.—A blank determination carried out on a sample of distilled water should give no precipitate. This was found to be the case as long as the solution was filtered

TABLE I.
Effect of Alcohol Concentration on Acetone Return.

Concentration of solution.	Alcohol concentration.	Acetone equivalent.	Weight of precipitate.	Acetone recovered.	Recovery.
vol. per cent	mg.	mg.	gm.	mg.	per cent
0.2	3.88	3.75	0.0763	3.8	101.5
0.5	9.70	9.38	0.1906	9.5	101.3
1.0	19.40	18.75	0.3636	18.2	97.0
2.0	38.80	37.50	0.7015	35.1	93.5
4.0	77.60	75.00	1.3023	65.1	86.7

hot. On cooling, a slight precipitate (white and not the usual brownish yellow) was deposited, undoubtedly arising from salts formed in the reaction. The practice of filtering hot was carefully adhered to. Gentle boiling during the reaction was important for violent heating caused a slight precipitation. The data in Table I show that different concentrations of alcohol yield varying returns of acetone. This was to be expected from the type of reaction employed. The experiments indicate that approximately 0.5 to 1.0 per cent solutions yield the maximum return of acetone.

Final Procedure in Detail.—The following solutions are required.

Copper Sulfate 20 Per Cent.—200 gm. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ dissolved in water and made up to a volume of 1 liter.

Mercuric Sulfate 10 Per Cent.—73 gm. of pure red mercuric oxide dissolved in 1 liter of 4 N H_2SO_4 .

Sulfuric Acid 50 Per Cent (by Volume).—500 cc. of acid, sp. gr. 1.835, added to an equal volume of water.

Calcium Hydroxide Suspension 10 Per Cent.—100 gm. of $\text{Ca}(\text{OH})_2$ in 1 liter of water.

Potassium Dichromate 5 Per Cent.—50 gm. of $\text{K}_2\text{Cr}_2\text{O}_7$ dissolved in water and made up to 1 liter.

Place 25 cc. of the sample (urine or preparations) in a 250 cc. volumetric flask containing 100 cc. of water. Add 50 cc. of CuSO_4

TABLE II.

Aqueous Solutions. Effect of Experimental Conditions on "Total Acetone" Determination.

Acetone recovered.	Weight of precipitate.	Concentration of $\text{K}_2\text{Cr}_2\text{O}_7$.	Heating period.	Concentration of H_2SO_4 .
mg.	gm.	cc.	min.	cc.
17.5	0.3507	5	90	10
17.2	0.3445	10	90	10
17.3	0.3470	15	90	10
18.6	0.3723	30	90	10
17.7	0.3544	40	90	10
18.3	0.3667	60	90	10
17.4	0.3474	40	30	10
17.4	0.3486	40	45	10
18.5	0.3702	40	60	10
18.3	0.3667	40	60	5
18.3	0.3653	40	90	15
17.7	0.3544	5	150	10
16.9	0.3390	5	120	10
18.7	0.3734	10	90	20
17.6	0.3515	20	90	40

In these determinations 19.4 mg. of isopropyl alcohol were present in the aqueous solutions, which is equivalent to 18.75 mg. of acetone. The concentration of HgSO_4 was maintained constant at 35 cc. of the 10 per cent solution. The acetone recovered equals the weight of precipitate divided by the factor 20 to give mg. of acetone.

solution and mix. Then add 50 cc. of the 10 per cent $\text{Ca}(\text{OH})_2$ suspension and shake well. Dilute to the mark and after allowing to stand for 30 minutes, filter clear. Interfering substances have now been removed and the filtrate is ready for the determination of total acetone.

Pipette 25 cc. of this filtrate containing not more than 0.1 per cent isopropyl alcohol (1.0 per cent for original urine) into a 500

cc. round bottom flask containing 65 cc. of water. Add 10 cc. of 50 per cent H_2SO_4 and then 35 cc. of the 10 per cent HgSO_4 . Place the flask under a reflux condenser and heat to boiling. After boiling has begun, add 40 cc. of 5 per cent $\text{K}_2\text{Cr}_2\text{O}_7$ solution through the top of the condenser. This gives a total volume of 175 cc., which is kept constant. Throughout the experimental work this total volume was maintained and the HgSO_4 concentration was held at 35 cc. of 10 per cent solution. The gentle boiling is continued for 1 hour, after which time the precipitate is collected in a Gooch crucible, dried at 100° , and weighed.

The weight of precipitate is divided by the factor 20 to give the acetone equivalent, a value representing both the preformed acetone and that derived from the isopropyl alcohol.

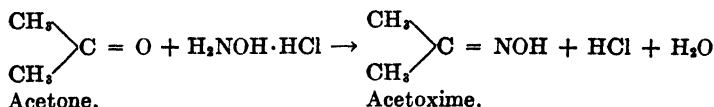
The material used in this experiment was a sample of anhydrous isopropyl alcohol¹ free from acetone. It was redistilled and the fraction boiling at 82.7° was employed. Its specific gravity was determined by the pycnometer method and found to be 0.786 at 20° . This value agrees with those in the literature for the anhydrous alcohol. The alcohol was delivered from calibrated pipettes under the surface of the liquid. All reasonable precautions to minimize the error due to volatilization were observed. The theoretical quantitative return of alcohol was never achieved but the data in Table II indicate the accuracy secured under the optimum conditions. The 1.0 per cent solution should yield a return of 18.75 mg. of acetone, while 18.5 mg. were actually recovered.

Determination of Preformed Acetone.

The reaction of hydroxylamine hydrochloride with acetone has long been known and has been generally regarded as a promising method for the accurate determination of the acetone present in commercial mixtures. It is surprising to find how little this method has actually been employed either for acetone or for higher ketones. This is due partly to certain inherent difficulties of the method, but, largely to lack of investigation of its application

¹ This was obtained from the Research Laboratories of the Standard Oil Company through the courtesy of Dr. C. O. Johns.

and possibilities. The reaction is represented by the equation in which hydrochloric acid is liberated on formation of the acetoxime.



This hydrochloric acid can be titrated with 0.1 N alkali with methyl orange. An indicator acting on the acid side of neutrality is necessary, and methyl red, alizarin red, brom-cresol green, and paranitrophenol have been tried, but methyl orange seems to be the most satisfactory one of the group. The above reaction is quantitative under proper conditions and since isopropyl alcohol gave no reaction on standing 20 hours with neutral hydroxylamine hydrochloride it seemed well suited to the determination of acetone in the presence of isopropyl alcohol. Hydroxylamine sulfate, $(\text{NH}_2\text{OH})_2 \cdot \text{H}_2\text{SO}_4$, may be used although in the following work the hydrochloride salt was used.

The hydroxylamine hydrochloride reaction was used for higher ketones by Bennett and Donovan (1922). These workers found it necessary to conduct the condensation reaction in stoppered bottles for a period of 2 hours to secure complete reaction. The concentration of the amine salt influences the rate of reaction (Mayer, 1919). A rapid estimation of acetone in industrial mixtures was based on this reaction by Marasco (1926), who found that 94.4 per cent is the maximum figure reached by the reaction and that the maintenance of approximate neutrality is a prerequisite to rapid condensation.

Procedure for Determination of Acetone in Presence of Isopropyl Alcohol.—This method was first tested on aqueous solutions of acetone and alcohol. The acetone samples were secured by redistilling a (U. S. P.) product and collecting the fraction which boiled at 56.5° and had a specific gravity of 0.792 at 20° . The alcohol used was the same sample as used throughout the preceding experiments. The acetone and alcohol were delivered under the surface of the water from calibrated pipettes. These solutions or aliquots were transferred to a Ladenburg distilling flask.

The flask was connected by a bent side arm to a vertical water condenser of convenient size. The end of the condenser was

attached to a calcium chloride tube dipping under the surface of the hydroxylamine salt solution and touching the bottom of the Erlenmeyer flask. The bulb part of the calcium chloride tube partially closed the mouth of the flask, an arrangement serving to minimize any loss of acetone by volatilization. A 100 cc. volume of a 0.4 per cent neutralized hydroxylamine hydrochloride solution was used to receive the distillate from the proper aliquot of the acetone solution. These aliquots never contained over 0.2 gm. of acetone. A few drops of methyl orange in the aqueous salt solution were useful in following the appearance of the acetone in the distillate. Most of the distillations required 4 minutes, although more dilute acetone aliquots required a little longer.

At the end of distillation the condenser was washed with distilled water and the washings added to the distillate. The salt solution containing the distillate and washings was immediately brought nearly to the neutral point with 0.1 N alkali. This approximate neutralization was repeated every few seconds until no appreciable quantity of acid was liberated in the course of 1 minute. It usually requires three additions of alkali, after which the true end-point is cautiously approached. It requires some experience before the end-point can be recognized quickly and accurately.

An aqueous solution containing 0.0921 gm. of acetone and 0.0915 gm. of isopropyl alcohol was distilled and the distillate collected in a neutral solution of hydroxylamine hydrochloride (0.4 per cent). On the basis of the sodium hydroxide used in the neutralization of the liberated acid 0.0930 gm. of acetone was recovered. Similar values were secured when no alcohol was present, proving that the alcohol did not react with the salt to liberate acid. It has been our experience that the salt solution when neutralized does not dissociate appreciably even on standing several hours. All of the hydrochloric acid titrated after condensation arises from the oxime reaction. Maintenance of approximate neutrality is important as the free acid will retard the condensation of the acetone with the hydroxylamine. This is undoubtedly due to the tendency to form the hydrochloride salt.

In case of foaming during distillation it was found permissible to use a few drops of caprylic alcohol. This alcohol does not react with the amine salt solution. Low concentrations of acetone do not yield the best returns and for this reason the

aliquots were chosen to contain between 0.1 and 0.2 gm., although the latter figure is the maximum acceptable under the conditions of the reaction.

Combined Determination Applied to Urine.

A urine sample containing 0.7831 gm. of acetone and 0.7781 gm. of alcohol (Table III) was analyzed. The acetone and alcohol were introduced with calibrated pipettes.

25 cc. samples of urine were pipetted into 250 cc. flasks and treated to remove interfering substances. 25 cc. samples of the filtrates were added to the oxidation and precipitation mixture

TABLE III.

Combined Determination Applied to Urine, Controlled Acetone and Alcohol Concentrations.

Theoretical amount of acetone, known concentration.	Acetone recovered by NH ₄ OH HCl method.	Acetone recovered.	Theoretical amount of alcohol.	Alcohol recovered by difference of two methods.	Alcohol recovered.
gm.	gm.	per cent	gm.	gm.	per cent
0.7831 *	0.7650	97.6	0.7781 *	0.7540	96.9
0.7831 †	0.7308	93.3	0.7781 †	0.7925	101.8
0.7831 ‡	0.6687	85.4	0.7781 ‡	0.8960	115.0

* 1 per cent solution, 0.7831 gm. acetone } in 100 cc. urine.
Volume per cent, 0.7781 " alcohol

† 0.5 per cent solution, 0.7831 gm. acetone } 200
Volume per cent, 0.7781 " alcohol

‡ 0.2 per cent solution, 0.7831 gm. acetone } " 500 " "
Volume per cent, 0.7781 " alcohol

and the total acetone content determined. The final dilution of the aliquot analyzed was $\frac{1}{40}$ the original concentration. In four check determinations the following weights of precipitates were obtained.

Acetone.				
0.7459 gm.	divided by factor	20	=	37.30 mg.
0.7460 " " " "		20	=	37.30 "
0.7483 " " " "		20	=	37.41 "
0.7484 " " " "		20	=	37.42 "

Average. 37.36 " $\times 40$ (dilution) = 1.4944 gm.
of total acetone.

To determine the preformed acetone, 10 cc. aliquots of urine were distilled into hydroxylamine hydrochloride as described in the foregoing sections. The titration values were as follows:

13.30 cc.	N 0.0948 NaOH
13.12 "	" " 0.0948 "
13.20 "	" " 0.0948 "
13.14 "	" " 0.0948 "

Average. 13.19 "

The acetone equivalent of 0.1 N alkali per cc. $\times 0.948$ (normality factor) $\times \frac{100}{94.4} = 0.0058$ gm. Thus 1.0 cc. of the N 0.0948 alkali was equivalent to 0.0058 gm. of acetone. The correction factor $\frac{100}{94.4}$ or 1.057 takes into account the fact that under optimum conditions the oxime reaction only reaches 94.4 per cent completion.

13.19 cc. $\times 0.0058$ gm. $\times 10 = 0.7650$ gm. of acetone in 100 cc. of urine.

1.4944 gm. total acetone from mercury precipitate.

0.7650 " acetone by $\text{NH}_2\text{OH} \cdot \text{HCl}$ method.

0.7294 " " from alcohol by difference.

$\frac{0.7294}{x} = \frac{58.06}{60.08}$; $x = 0.7540$ gm. alcohol, equivalent to 0.7294 gm. acetone.

Alcohol.

0.7781 gm. alcohol, theoretical.

0.7540 " from difference of two methods.

96.9 per cent recovery.

Acetone.

0.7831 gm. acetone, theoretical.

0.7650 " by $\text{NH}_2\text{OH} \cdot \text{HCl}$ method.

97.6 per cent recovery.

SUMMARY.

A method for the determination of isopropyl alcohol alone or in the presence of acetone has been developed and has been applied to the analysis of urine.

Certain requirements as to the concentration of alcohol and acetone and the optimal experimental conditions are discussed.

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AN ENZYMATIC METHOD FOR THE DETECTION AND ESTIMATION OF TYROSINE IN URINE.

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INTRODUCTION.

The crystalloscopic method commonly used for the detection of tyrosine in urine concentrates was first employed by Frerichs and Städeler (1) when they observed this amino acid in a case of acute yellow atrophy of the liver. This method and its modern modifications (2, 3) is laborious, time-consuming, and of doubtful reliability.¹ We undertook to replace it by a method which would permit of quantitative as well as of qualitative statements and which might also be applied as a tyrosine test in other biological fluids (5).

The enzymatic oxidation of tyrosine was first employed by Bertrand (6) as a qualitative test. The enzyme tyrosinase is found in certain fungi, in the meal-worm, in potatoes, etc. The mechanism of its action has recently been elucidated by Raper (7). The primary phase of the reaction, the formation of a rose-colored substance, is entirely dependent on enzymatic activity; the subsequent non-enzymatic formation of melanin adds to the sensitivity and to the specificity of the reaction.

The present application of this reaction as a dependable qualitative and quantitative test for tyrosine is made possible by the preparation of a fairly stable dry standardized tyrosinase described below and the recognition of a threshold reaction. The red substance affords an excellent standard for colorimetric comparison in the early stages of the reaction, while the violaceous end stage permits the identification and colorimetric comparison even in dark urines.

¹ A thorough critical survey of other tyrosine methods was recently made by Mitchell and Hamilton (4).

TABLE I.
Standardization of Enzyme. Demonstration of Relationship between Minimal Enzyme and Substrate Concentrations for Threshold Reactions.

Final volume in each tube equals 5 cc. of phosphate buffer, pH 6.8, as diluent.

Tyrosine. mg.	Enzyme, mg	2	2.5	3	5	7.5	8	10	11	12.5	15	17.5	25	35	45	55
0.2	Rose color, <i>min.</i> Melanin visible.					No. 0		No. 0		No. F. V.	20 V.		14 V.	13 V.	12 V.	11 V.
0.5	Rose color, <i>min.</i> Melanin visible.			No. 0	No. F. V.		No. F. V.				12 V.		9 V.	7 V.		
1.0	Rose color, <i>min.</i> Melanin visible.	No. 0	No. F. V.						4 V.			3 V.	3 V.	2 V.		

F. V. = faint violet; V. = violet.

*Quantitative Relations between Tyrosine and Tyrosinase.
Standardization of Enzyme.*

While the time of appearance of the rose-colored substance under constant conditions may be used for measuring the purely enzymatic portion of the oxidation (Table I), a more sensitive method for the evaluation of minute amounts of tyrosine is afforded by the constancy of the threshold amount of visible melanin.

The method for the estimation and standardization of tyrosinase has likewise been based on the empirical relationship between the concentrations of enzyme and substrate necessary for melanin formation. The product of these two concentrations was found constant within ample limits (Table I). However, irrespective of an excess of enzyme, concentrations of tyrosine less than 0.002 per cent fail to form melanin.

The "unit" concept as developed by Willstätter and his school (8) in the study of numerous other enzymes has hitherto not been applied to the measurement of tyrosinase. We suggest as a practical tyrosinase unit that minimal amount of enzyme which will form a visible amount of melanin within 24 hours when reacting with 0.2 mg. of tyrosine in 5 cc. of a phosphate buffer solution of pH 6.8 at room temperature. The procedure for the determination of this unit is described in the experimental part. The definition of a tyrosinase unit permits the assay of preparations, the comparison of various methods of separation, and purification, and the determination of the rate of deterioration of the enzyme.

A unit has been found in approximately 12.5 mg. of an active crude preparation. 100 cc. of active potato juice yielded up to 70 units. The tyrosinase value of a preparation is defined as the number of units contained in 100 gm. of the preparation; thus the tyrosinase value of an active enzyme has been found to be about 8000.

Sensitivity of Test; Inhibitory Factor in Urine.

When the reaction is carried out under standard conditions in the presence of an excess of oxygen and enzyme, tyrosine can be detected in solutions of free tyrosine in a minimal concentration between 0.004 and 0.002 per cent or 1:25,000 to 1:50,000 (Table II).

The application to urine disclosed that in this medium the sen-

sitivity of the test was reduced. Tyrosine was added to normal and pathological urines and the test carried out as with pure solutions. The minimum concentration detectable in undiluted urine and in urine diluted 1:10 parts was 0.0125 per cent or 1:8000 (Table II). This elevation of the threshold was constant. In dilutions of 1:25 of urine the original threshold is regained. In comparison, the Frerichs-Städeler method will detect approximately a minimum of 200 mg. in 400 cc. of original urine or 0.050 per cent.

The reduction of the sensitivity of the test in urine is due to the presence of a substance inhibitory to the action of the ferment.

TABLE II.

Melanin Threshold in Buffer Solution and in Urine.

50 mg. of enzyme (tyrosine value = 8000 units) in each tube.

Tyrosine.		Control.		Urine.		
	0.05 per cent solution.	Buffer solution.	Color in 24 hrs.		Buffer solution.	Color in 24 hrs.
mg.	cc.	cc.	No.	cc.	cc.	
0.05	0.1	4.9	No.			
0.1	0.2	4.8	Faint violet.			
0.2	0.4	4.6	" "	4.0	0.6	0
0.3	0.6			4.0	0.4	0
0.4	0.8	4.2	Violet.	4.0	0.2	0
0.5	1.0			4.0	0	Black.
0.6	1.2	3.8	Violet.	3.8	0	"
0.8	1.6	3.4	"	3.4	0	"
1.0	2.0	3.0	"	3.0	0	"

Excess amounts of urea, uric acid, bile, or indican have been found not to be responsible for this inhibitory effect. The exact nature of the inhibitory substance awaits further investigation.

Atypical Reactions.

Tyrosinase reacts with most substances containing a phenolic OH group. With few exceptions these substances are derived from the decomposition of tyrosine in the body. While the primary color reactions varying between yellow and red have been reported with such substances as pyrogallol, polypeptide chains containing tyrosine (9), aromatic hydroxy amines and hydroxy acids

(6), tryptophane (10), sodium salicylate, adrenalin (1:25,000), hydroquinone (1:1000), and *o*-, *m*-, and *p*-cresol (1:50,000) the formation of melanin is confined to tyrosine and dioxyphenylalanine. A remarkable stereochemical specificity permitting the distinction of tyrosine even from its isomers *o*- and *m*-hydroxyphenylalanine on treatment with tyrosinase, on the basis of the formation of melanin has been noted (10).

That the phenols, a great part of which is excreted as *p*-cresol (11), do not occur in sufficient quantities in the free state to be detected by our method, is affirmed by the fact that a positive test for these substances was not obtained in a series of 50 normal and twenty-five pathological urine samples. This is all the more significant as the threshold for the detection of the three cresols in dilutions of urine up to 1 in 10 parts is approximately 1:50,000. *p*-Cresol is recognized by the intensity of the red color and the rapidity of its formation. Concentrations of tyrosine giving as intense an initial red coloration turn brown in about an hour and black within 7 hours. When cresol is added to tyrosine its presence is apparent by the brown coloration with a persistent rose background. With concentrated tyrosine solutions the dark coloration effaces the red. The quantitative determination under these conditions may be attempted by steam distillation of the phenols after removal of albumin. Subliminal concentrations of *p*-cresol and tyrosine were found not to be supplementary.

EXPERIMENTAL.

Preparation of Enzyme.—An economical source of enzyme is potato juice. Young potatoes yield more active enzyme than old ones, and juice from unpeeled potatoes is richer than from peeled according to Haehn and Stern (12). The Idaho potato is a poor source of tyrosinase because of its high starch content.

The following method of preparing tyrosinase in stable dry form is far from efficient in regard to purity and yield, as the elimination of inert material is offset by loss of enzymatic activity during the various steps of the preparative process.

The potatoes are washed and scrubbed in cold water, minced, and put through a meat press. The juice is strained through several layers of gauze and the juice expressed from the strained material, added thereto. Starch is permitted to settle out and

after allowing the juice to stand $\frac{1}{2}$ to 1 hour at room temperature it is centrifuged for 5 to 10 minutes. The supernatant fluid is then precipitated with an equal volume of 95 per cent alcohol, the precipitate taken up with water, and reprecipitated with alcohol. The gray precipitate is spread in thin layers on porcelain dishes and allowed to dry *in vacuo* over sulfuric acid at room temperature for 24 to 72 hours. The tyrosinase is "ripe" when it becomes glistening jet-black in color. The enzyme thus prepared gives negative sugar reactions with Benedict's solution and a negative test for pentoses with Bial's reagent, negative tests for peroxidase with benzidine and with α -naphthol, and a negative test for iron.

A preparation will retain its activity for months but for the present method new stock is best prepared every fortnight as needed. It is stored in sealed tubes or vials sealed with paraffin. Przibram and Dembowski (13) have shown that in the absence of O_2 it is not sensitive to light.

Determination of Unit of Tyrosinase.—The raw juice and the dry preparation are titrated in serial dilution to determine the moist and dry units respectively. The reaction mixture consists of 0.2 mg. of tyrosine in a final volume of 5 cc. of buffer and varying amounts of juice or of dry enzyme weighed at the outset into test-tubes with side arms. The tyrosine solution is best prepared as needed, 0.05 per cent in 0.2 M phosphate buffer solution pH 6.8 (equal parts of KH_2PO_4 , 27.23 gm. per 1000 cc. and $Na_2HPO_4 \cdot 2H_2O$, 35.62 gm. per 1000 cc.). The samples are aerated for 120 minutes by a current of air drawn through capillary tubes to insure constant agitation and equal distribution of enzyme. The pressure in the system is equalized through a central wash bottle whose stopper is perforated to admit a sufficient number of connections to the side arms of the test-tubes, also a vent and an outlet. The connections are provided with screw clamps. A drop of caprylic alcohol is added to each sample as an antifoam.

The final reading is made in 24 hours against a white background. The color varies from faint violet to slate color and the threshold tube may be identified in doubtful instances in the colorimeter. The experiment with 0.2 mg. of tyrosine shown in Table I illustrates the arrangement for the determination of the enzyme unit.

Calculation of Yield.—195 cc. of potato juice containing 9.65 gm. of total solids yielded 1.43 gm. of dry preparation, 7.3 gm. per 1 cc.

Preliminary titration of the raw juice indicated that a "moist unit" was contained in 0.2 cc. or the equivalent of 9.9 mg. of dry solids. The titration of the dry preparation gave 12.5 mg. for 1 "dry unit." The tyrosinase value was therefore 8000. The total yield was $\frac{1.43}{0.0125}$ gm. = 115 units.

Threshold.—The preparation employed in the experiment in Table I had a tyrosinase value of 8000. On increasing the tyrosine concentration from 0.004 per cent to 0.01 and 0.02 per cent the amount of enzyme required for melanin formation was reduced from 12.5 mg. (1 unit) to 5.0 and 2.5 mg. (0.4 and 0.2 units, respectively). The product of the minimal concentrations of enzyme \times substrate which will give a threshold reaction is constant within these limits. Quantities of enzyme less than 2 mg., consisting of very few particles, introduce a source of error as variable, considerable amounts are temporarily withdrawn from the reaction during aeration. If these minute amounts of enzyme below 2 mg. could be depended upon in the reaction, the constancy of the above product might be demonstrable up to saturated solutions of the amino acid. The demonstration of this relationship was repeated with other preparations of the same and of lower tyrosinase value.

Determination of Tyrosine in Urine.—For the accurate determination of daily output a specimen is carefully collected for 24 hours. While fresh urine samples are desirable, the test may be performed on old urine as well; a positive test was obtained in a specimen a fortnight old. If the total quantity of urine for 24 hours exceeds 1000 cc., a small portion is concentrated on the water bath to a volume corresponding to 1000 cc. total. A pH of 6.8 is adjusted by dilute HCl and Na_2CO_3 solution. Correction must be applied for these changes in volume. About 25 cc. are filtered clear.

The reaction is carried out in test-tubes, 150 \times 20 mm. with side arms, of equal mold, size, and thickness of glass. The final volume is 5 cc. for all tubes; the dilutions are made with 0.2 M buffer solution. The amount of enzyme is an excess over the unit and must be fairly constant in all tubes. It has been found expedient to employ the content of a 2 grain No. 5 gelatin capsule used for dispensing pharmaceutical preparations as a convenient

measure for this purpose. These capsules, filled, hold approximately 50 mg. For dark colored urine we recommend the use of the contents of two capsules.

A preliminary test is made to detect the presence and the approximate range of concentration. The plan for this test is represented in Table III. To control Tubes 1, 3, 5, and 7, 0.5 mg.

TABLE III.
Plan for Standard Preliminary Test.

Tube No.....	7, control.	6	5, control.	4	3, control.	2	Control, Enzyme 1, boiled.
Tyrosine in buffer (1 cc. = 0.5 mg.) cc	1		1		1		1
Urine, cc.....	0.5	0.5	1	1	4	4	4
Buffer pH 6.8, cc.....	3.5	4.5	3	4	0	1	0
Enzyme in excess, <i>ma</i>	50	50	50	50	50	50	50

TABLE IV.
Example for Final Test (Case 3).

Calculation.— $2x < 0.5$ mg. (Tube 6 negative)

$2.5x \geq 0.5$ " (" 4 positive)

Hence $x < 0.25$ mg. and ≥ 0.2 mg.

Tyrosine concentration = 0.02 – 0.025 per cent or 20 to 25 mg. per 100 cc. of urine.

Tube No.....	7, control.	6	5, control	4	3, control.	2
Tyrosine in buffer, (1 cc. = 0.5 mg.) <i>mg.</i> ..	0.5	0	0.5	0	0.5	0
Urine, cc.....	2	2	2.5	2.5	3	3
Buffer pH 6.8, cc.....	2	3	1.5	2.5	1	2
Resulting color, <i>min.</i> ..	Dark, 50	No.	Rose, 40	Dark, 60	Rose, 30	Dark, 60
Relative intensity. ..	2	0	3	1	4	1+

or 1 cc. of a fresh clear 0.05 per cent solution of tyrosine is added. Tube 1 contains the same constituents as Tube 3 save that the enzyme is boiled for 5 minutes on the water bath in buffer solution before addition of the urine. This control aids in the detection of minimal amounts of tyrosine in dark specimens. The tubes are aerated for 120 minutes as described for the determination of the enzyme unit. The entire test is carried out at room temperature and observations are made at 1, 3, 7, and finally at 24 hours.

A positive result in Tube 2, while Tubes 4 and 6 are negative, signifies the presence of tyrosine in concentrations varying between 0.0125 and 0.05 per cent. A conclusive titration is made within this short range following the same procedure as in the preliminary test but varying the dilution of urine between 1:5 and 4:5 (see Table IV), and the dilution in which the reaction becomes extinct is taken as a basis for the calculation. In the minimal concentration of 0.0125 per cent the red phase is not visualized in the presence of urine, and the dusky phase of melanin is the first phase in evidence. A disappearance of the dark coloration obtained with minimal amounts of tyrosine is occasionally observed after about 12 hours. This clearing proceeds from the bottom upward, leaving a dark layer in contact with the air.

A positive result in Tubes 2 and 4, while Tube 6 is negative, signifies the presence of 0.05 to 0.1 per cent in the urine. In this event a titration is made with dilution of 1 part of urine in 5 to 10 parts. There need be little loss of time in setting up the second titration since the indications become apparent in about 1 hour in the preliminary test.

If Tube 6 is also positive, at least 0.1 per cent tyrosine is present and the final test is carried out in higher dilutions. In dilutions of 1:25 and higher the inhibitory effect of urine disappears and a concentration of 0.004 per cent can be detected. In this case only 0.2 mg. is added to the controls.

Under the constant conditions of this method both the intensity of the red color and of the violaceous black may be accurately compared. The method of calculation is best illustrated by the concrete cases, Nos. 3 and 4 of the protocols following. The final test permits establishing the upper and lower limit of the tyrosine concentration of the specimen from the last positive tube and the negative tube next to it. This range is narrowed by comparing the intensity of color in the several tubes with and without known amounts of tyrosine added.

Detection of Subthreshold Amounts.—In the event that Tube 2 (Table III) is negative, the sensitivity of the test may be increased 3 to 4 times by the addition of subthreshold quantities of tyrosine to subthreshold concentrations present to realize a threshold reaction. As a control the adsorption and destruction of tyrosine

on blood charcoal (14) was used for the removal of tyrosine possibly present in subthreshold amounts.

Experimental Data for Pathological Urines.

The findings in five positive and otherwise significant cases in a series of twenty-five pathological urine samples from cases associated with jaundice of varied etiology are here cited.

Case 1.—In a case of cirrhosis of the liver with ascites in a single urine specimen of few cc., a positive result was obtained by the present method, while the routine method was negative. The quantity present was more than 12.5 mg. and less than 50 mg. per 100 cc. of urine or a concentration of tyrosine between 0.0125 and 0.050 per cent. Owing to the fact that the patient left the hospital against advice, a conclusive test could not be made.

Case 2.—This was a case of subacute yellow atrophy of the liver verified by necropsy. In view of the negative findings by the present method, the crystals obtained by the routine method which answered the description of tyrosine crystals were found on purification to be readily soluble in cold water and gave a negative Millon test.

Case 3.—Hospital No. 299,505, Mar. 7, 1929. Diagnosis: metastatic carcinoma of liver; carcinoma of stomach, confirmed by x-ray examination. A single specimen of 50 cc. of orange-colored urine, containing bile but no albumin, was examined. The Frerichs-Städeler test in this case was negative. In a preliminary test set up according to the scheme of Table III, no melanin was found in Tubes 6 and 4, with a positive result in 35 minutes in Tube 2 which was slightly darker than the control Tube 5, but lighter than Tube 3. Thus the amount of tyrosine in 1 cc. of urine is smaller than 0.5 mg. On the other hand, 4 cc. of urine contained more tyrosine than 1 cc. of urine plus 0.5 mg. of added tyrosine; hence 3 cc. of urine contained more than 0.5 mg. of tyrosine and the limits per 1 cc. are 0.16 to 0.5 mg. of tyrosine. The final test and calculation are illustrated in Table IV.

Case 4.—Hospital No. 300,298, Mar. 11, 1929. Diagnosis: acute yellow atrophy of liver. Because of urinary incontinence, the total daily output could not be determined. However, in a 12 hour period, at least 525 cc. of urine of deep yellow color, containing bile but no albumin, were voided. The Frerichs-Städeler test was negative. The preliminary test according to Table III revealed the presence of tyrosine in the highest dilution. As Tube 4 (1 cc. of urine) showed a darker color than the control Tube 7 (0.5 cc. of urine + 0.5 mg. of added tyrosine), the amount $\frac{x}{2}$ (tyrosine in 0.5

cc. of urine) must be greater than 0.5 mg. Thus a final test with 0.2, 0.1, and 0.05 was made. In these dilutions of urine of 1:25 and greater the inhibitory effect disappears and a concentration of 0.004 per cent tyrosine is detected. Thus the controls were set up with 0.2 mg. of tyrosine only:

0.05 cc. urine negative.

0.1 " " positive but weaker than 0.05 cc. urine + 0.2 mg. tyrosine added.

Therefore $0.05x < 0.2$ mg. and x is < 4 mg. per 1 cc.

Since 0.2 cc. of urine gave the same color as 0.1 cc. + 0.2 mg. of tyrosine added, the concentration of tyrosine can be accurately estimated to be 0.2 mg. in 0.1 cc. or 200 mg. in 100 cc. of urine.

With this knowledge the crystalloscopic method was repeated, the precipitates being carefully washed with boiling water and a fair amount of atypical crystals recovered which, on purification with ammoniacal alcohol, gave a positive Millon test and were insoluble in cold water. Morphologically alone these crystals could not be identified positively as tyrosine (*cf.* (15)).

Case 5.—In a case of hepatitis in a patient with cerebrospinal lues the routine method yielded some atypical crystals which proved to be tyrosine by the Millon test, the present test in this case being negative. Analysis of this failure, the only failure of the method thus far encountered, discloses a possible explanation. The total volume voided in 24 hours was 2000 cc. This case emphasizes the importance of concentrating dilute urine to 1000 cc. to bring minimal amounts of tyrosine within the range of detection. Dilution is not a factor in the Frerichs-Städeler method provided at least 0.200 gm. is present.

The maximum concentration thus far encountered was that observed in Case 4, 0.20 per cent. At no time has the special method for the detection of amounts below 0.0125 per cent been positive. At no time has a normal urine sample in a series of 50 normal specimens yielded a positive result by the present method.

Comparison of Qualitative Results of Crystalloscopic and Biochemical Methods.

When the results obtained by the biochemical method are compared with those obtained by the Frerichs-Städeler method in a series of twenty-five unselected pathological urine samples from cases of jaundice of varied etiology summarized below, it is apparent that the present method is more sensitive and dependable, and in addition permits of the estimation of the amount of tyrosine present.

Findings negative, both methods.....	20 cases.
“ positive, Frerichs-Städeler method; disproved by biochemical method (Case 2).....	1 case.

Findings negative, Frerichs-Städeler method and Millon test; found by biochemical method (Cases 1, 3, 4*)..... 3 cases.
Findings positive, Frerichs-Städeler method and Millon test; not found by biochemical method (Case 5)..... 1 case.

* Confirmed afterward by Frerichs-Städeler method.

SUMMARY.

A method for the preparation of tyrosinase in dry stable form is described. A practical method for the standardization and estimation of tyrosinase is outlined.

A biochemical method is described for the detection of free tyrosine in untreated urine based on the enzymatic oxidation of this amino acid. Its principle is the comparison of the primary red and secondary brown and black phases of the reaction and the recognition of a melanin threshold when an adequate amount of enzyme interacts with a minimal concentration of 0.0125 per cent tyrosine in urine.

The presence of an inhibitory factor in urine which reduces the sensitivity of the test is demonstrated. Substances giving atypical reactions are enumerated and the criteria of their differentiation, especially that of *p*-cresol, stated. The applicability of our method to the detection of tyrosine in other pathological body fluids is mentioned.

The advantages of the biochemical over the crystalloscopic method as a qualitative method involve economy of time and labor, higher specificity, sensitivity, dependability, and its application directly to untreated urine. In addition, it permits the quantitative evaluation of tyrosine.

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THE INFLUENCE OF INSULIN AND EPINEPHRINE ON GLYCOGEN FORMATION IN THE LIVER.

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When insulin was injected during glucose or fructose absorption, the deposition of liver glycogen was markedly retarded at all periods up to 5 hours after the injection (1). Barbour, Chaikoff, Macleod, and Orr (2) confirmed this observation. In the above experiments the blood sugar of the animals receiving glucose plus insulin remained at a lower level than that of the animals receiving glucose alone. This might have been one of the factors responsible for the slow rate of glycogen deposition in the liver after insulin injections. It also seemed possible that an action of insulin favoring deposition of liver glycogen might not be observable at low blood sugar levels. The present experiments were undertaken in order to test these two possibilities.

EXPERIMENTAL.

The aim was to maintain the blood sugar at a high level during the period of insulin action. The best way in which this could be accomplished was by means of an intravenous injection of glucose into nephrectomized rats. The animals were fasted for 24 hours previous to the experiments. This reduces the liver glycogen to a level of 0.1 to 0.2 per cent.¹ Amytal (7 mg. per 100 gm. rat) was injected intraperitoneally and both kidneys were removed by

¹ The glycogen content of the liver of young rats after a fasting period of 24 hours is given as follows:

Karczag *et al.* (3)..... 0.160 ± 0.09 per cent (average of 24 experiments).

Barbour *et al.* (2).... 0.160 ± 0.06 per cent (average of 24 experiments).

Cori and Cori (4).... 0.170 ± 0.05 per cent (average of 24 experiments).

the lumbar route. Immediately after the operation a subcutaneous injection of insulin (4 units per 100 gm. rat) was made and

TABLE I.

Deposition of Liver Glycogen during 2 Hours of Glucose Injection in Rats under Amytal Anesthesia.

Body weight.	Per 100 gm. body weight.			Liver glycogen.	Blood sugar.	Remarks.
	Glucose injected.	Weight of liver.	Glycogen in liver.			
gm.	mg.	gm.	mg.	per cent	mg. per cent	
150	846	3.62	88	2.43	576	
174	740	3.98	105	2.64	741	
160	800	3.86	113	2.93	864	
154	800	3.78	101	2.67	815	
188	800	3.41	49	1.44	504	
197	778	3.46	121	3.50	512	
170	794	3.68	96	2.60	669	
150	862	3.36	51	1.52	375	Insulin injected.
170	734	3.78	43	1.13	346	
159	800	3.70	84	2.27	326	
167	802	3.54	22	0.62	252	
148	800	3.66	47	1.28	230	
159	800	3.61	49	1.36	306	
158	590	3.54	54	1.52	562	
152	594	3.72	72	1.93	355	
149	590	3.74	46	1.23	428	
160	590	3.86	45	1.16	420	
151	564	3.34	47	1.41	322	
152	586	3.67	53	1.45	417	
148	790	3.32	84	2.53	1290	Epinephrine injected.
156	796	3.47	71	2.04	1220	
186	800	3.26	35	1.07	1220	
160	800	3.64	42	1.15	1270	
177	800	3.52	60	1.70	1250	
165	797	3.44	58	1.69	1250	

glucose was injected intravenously for 2 hours, the rate being kept constant. Burette readings were made every 2 minutes. Depending on the weight of the rat, from 5 to 7 cc. of a 20 per cent

glucose solution were given in 2 hours. This corresponds to a rate of infusion of 400 mg. of glucose per 100 gm. rat per hour. The temperature of the animals was controlled at frequent intervals and was maintained as evenly as possible, heat being supplied by an electric bulb. In a second series of experiments the conditions were the same with the exception of the insulin injection and in a third series epinephrine (0.02 mg. per 100 gm. rat) instead of insulin was injected. Finally, it seemed desirable to supply glucose at a rate which would keep the blood sugar at the level obtained with larger amounts of glucose plus insulin. In all cases blood sugar and liver glycogen were determined after 2 hours of glucose injection, the former by means of the Hagedorn and Jensen and the latter by means of Pflüger's method. The results are shown in Table I.

The following previous observations are of interest in conjunction with the rate of infusion used in the experiments in Table I. When glucose was supplied at a rate of 250 mg. per 100 gm. rat per hour, no glycosuria resulted and the blood sugar level remained below 200 mg. per cent (5). When glucose was supplied at a rate of 450 mg. per 100 gm. rat per hour, from 13 to 29 per cent of the amount infused was eliminated in the urine (5). This large excretion made it desirable to remove the kidneys in the present experiments. It will be noted in Table I that the average blood sugar was 669 mg. per cent and that an average of 96 mg. (or 2.6 per cent) of glycogen was deposited in the liver when glucose was supplied at the rate of 400 mg. per 100 gm. rat per hour for 2 hours. This is decidedly more glycogen than was formed in unanesthetized rats during glucose absorption from the alimentary canal. In such animals in which the blood sugar rose to 150 mg. per cent during the period of absorption and in which 185 mg. of glucose were absorbed per 100 gm. rat per hour, an average of 68 mg. (or 1.94 per cent) of liver glycogen was formed in 2 hours (6). According to Hines, Leese, and Barer (7) amytal has a depressing influence on glycogen formation in the liver of dogs. In the present experiments this effect of amytal is apparently counteracted to some extent by the high blood sugar level. The rate of glycogen formation in the liver seems to be influenced by the blood sugar level; otherwise it would be difficult to explain why the animals under the less favorable conditions of general anesthesia form more liver glycogen

than normal rats after a glucose meal. When only 300 mg. of glucose per 100 gm. rat per hour were injected (Table I), the inhibitory action of amytal became clearly noticeable since in this case the animals formed less liver glycogen than the unanesthetized rats during glucose absorption.

The animals receiving insulin deposited less liver glycogen than the animals receiving the same amount of glucose but no insulin (Table I). Though a surplus of sugar was available and a high blood sugar level was maintained, insulin failed to show any action accelerating synthesis of liver glycogen. On the other hand, the blood sugar level of the insulinized animals was again lower than in those receiving glucose but no insulin and this might have been responsible for the decrease in deposition of liver glycogen. In order to test this possibility, a group of rats was given less glucose with the object in view of maintaining the same blood sugar level as in the experiments with insulin. This was not quite successful since the blood sugar was somewhat higher (417 against 306 mg. per cent). Nevertheless, the result is quite clear cut, showing that the difference in deposition of liver glycogen disappears when the blood sugar of uninjected and insulinized animals is kept at a similar level. Considering what small fractions of the total amounts injected are deposited as liver glycogen (9 and 6 per cent respectively), the blood sugar concentration and not the amount of glucose administered must be regarded as important for the rate of glycogen deposition in the liver.

Several factors seem to be involved in the low glycogen content of the liver after insulin injections. The disappearance of liver glycogen which is observed in fasting animals after insulin injections, is due to a mobilization of liver glycogen, elicited by the sub-normal blood sugar level. Since in previous experiments on rats during glucose absorption the blood sugar fell below the normal level after the insulin injection, mobilization of liver glycogen may have been partly responsible for the low glycogen content of the liver. In the present experiments in which hypoglycemia was avoided, another factor came to light; namely, a dependence of glycogen formation in the liver upon the blood sugar concentration. Since the blood sugar of animals injected with insulin is always lower than that of control animals receiving the same amount of glucose, the difference in deposition of liver glycogen is also due to some extent to the difference in the blood sugar level.

In the experiments with epinephrine the blood sugar rose to extremely high values and less liver glycogen was found than in the control animals receiving the same amount of glucose (Table I). The smaller glycogen content of the liver is probably the result of a mobilization of part of the newly formed glycogen. It is to be assumed that such a mobilization is continuously taking place during epinephrine action but its occurrence cannot always be demonstrated by means of glycogen determinations in the liver. In previous experiments on unanesthetized animals 2, 3, and 4 hours after epinephrine injection new formation of liver glycogen from blood sugar and lactic acid (the latter derived from muscle glycogen) overbalanced glycogen mobilization, with the result that more liver glycogen was found than in the control animals (6). In the present experiments in which an anesthetic was used, mobilization of liver glycogen was facilitated and thus exceeded the synthetic process.

The previous contention that the action of epinephrine on the peripheral tissues (muscles) plays a large rôle in the production of a sustained hyperglycemia is also illustrated in the present experiments. Obviously, the difference in deposition of liver glycogen of 38 mg. cannot explain the difference in blood sugar of 580 mg. per cent (Table I). It seems difficult to interpret this marked rise in the blood sugar level in any other way than by a decreased utilization of blood sugar in the muscles. The anesthetic seems to intensify this action of epinephrine. A factor which might be of importance in this phase of epinephrine action is an inhibitory effect of epinephrine on the insulin production of the pancreas.²

SUMMARY.

1. Deposition of liver glycogen during a continuous intravenous injection of a large excess of glucose was determined in nephrectomized rats under amytal anesthesia.

2. For an equal quantity of glucose injected, the insulinized animals showed a blood sugar level and a glycogen content of the liver approximately one-half of that of the control animals.

3. When a similar blood sugar level was maintained in control and insulinized animals by injecting less glucose into the former

² This possibility was suggested to the writer in a private communication by Dr. A. R. Colwell (Boston).

than into the latter, there was no difference in the amount of glycogen deposited in the liver of the two groups of animals. It was concluded that the blood sugar concentration is a factor determining the rate of glycogen formation in the liver.

4. When epinephrine was injected, less glycogen was formed than in the control animals receiving the same amount of glucose, in spite of the fact that the blood sugar level of the former animals was nearly twice as high as that of the latter. The low glycogen content of the liver was ascribed to a preponderance of mobilization over new formation of liver glycogen in anesthetized animals receiving epinephrine injections.

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STUDIES ON THE INORGANIC CONSTITUENTS OF THE BLOOD OF NORMAL AND PARATHYROIDECTOMIZED DOGS.

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Numerous efforts have been made to explain the genesis of parathyroid tetany on the basis of the interrelations of the various blood constituents. While it scarcely seems possible that a solution of the problem can be reached in that way, because blood composition is not always a true index of tissue changes, it is, nevertheless, of importance that these relationships be well established and defined.

To this end we have made simultaneous determinations of five constituents of blood plasma. Ca, inorganic P, Na, K, and Mg.

Previously, Hastings and Murray (1) had made simultaneous studies of a number of blood constituents in a few dogs, but these were not carried through for any length of time in order to determine whether there was any adaptation or readjustment to parathyroid deficiency after the acute postoperative period was past. Subsequently, Salvesen, Hastings, and McIntosh (2) made some simultaneous determinations of the same five constituents in dogs during oral administration of phosphates until tetany resulted, producing clinical symptoms identical with those resulting from parathyroid deficiency. Very recently, Greenwald (3) has reported decreased excretion of sodium and potassium by dogs after parathyroidectomy, which he suggests is due to interference with renal function as a result of decreased calcium concentration. In this paper, he does not report the blood concentrations of sodium and potassium.

Very large dogs were selected and confined to a stock diet which consisted of ground beef heart and Puppy Meal,¹ 3:1, and distilled water. Blood samples were drawn, by cardiac puncture,

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¹ Prepared by Dog Food Company, Needham Heights, Massachusetts.

into tubes containing heparin. About 20 cc. of blood were necessary to secure enough plasma for a complete series of check determinations. Normal dogs were bled approximately 19 hours after feeding. With few exceptions this was also the interval for operated dogs. In the exceptional cases, the interval was always greater than 19 hours. Only occasionally was any one animal bled more often than twice a week, a procedure which reduced the possibility of any pronounced change in concentration of any of these constituents resulting from repeated loss of blood.

Several months were occupied in studying the methods of analysis and the reactions of normal dogs to confinement. Pre-operative observation periods ranged from 2 weeks to about 3 months. Sixteen animals were operated upon. Three of these died before any postoperative observations could be made. The others lived for periods ranging from 3 days to 6 weeks. No treatment was employed except that parathormone was occasionally administered in minimum doses that would prolong life when the animals were in tetany. Only rarely was the dose large enough to restore calcium and phosphorus to normal concentrations.

Methods.

Calcium was determined by the Clark and Collip (4) modification of the Kramer-Tisdall (5) method, except that the precipitation was prolonged overnight. Inorganic phosphorus determinations were made by the method of Fiske and Subbarow (6).

The determination of sodium was based on the method of Kramer and Gittleman (7), with some modifications introduced by Kerr (8) and Rourke (9). Some difficulty was experienced with this method. It was found necessary to adopt a definite procedure which gave consistent results and good checks, and to adhere closely to the same technique thereafter, since we were concerned less with the absolute values than with changes in the concentration of the constituents under observation. Blank determinations on the reagents at the same concentrations as in actual analyses required 0.5 cc. of 0.05 N thiosulfate solution, so that this correction was made on all readings. 5 cc of pyroantimonate solution were added to 1 cc. of heparinized plasma in a 15 cc. Pyrex tube reserved for sodium determination only. These

tubes were rubbed with glass rods, a separate rod being reserved for each tube and carried through with it to the titration. After cooling, cold alcohol was added and allowed to stand 30 minutes before centrifuging. The rest of the technique was carried out as described by Rourke.

Potassium determinations were made by the Kerr (8) modification of the Kramer-Tisdall method, with some slight further modifications. Porcelain dishes were used for evaporating the protein-free filtrate and the nitric acid added later. The evaporation was accomplished in an oven kept at 110° . A quantity of fresh NaOH was placed in the oven to absorb the acid fumes. After the addition of sodium cobalti-nitrite, the tubes were allowed to stand overnight for precipitation.

Magnesium determinations were carried out by the method of Denis (10) with some minor changes. 4 cc. of the supernatant fluid from the calcium precipitation were taken, instead of 2 cc., as the original method calls for, and 1 cc. of the ammonium phosphate-ammonium hydroxide mixture was added, giving the same proportions used by Denis. Cresol red showed that the proper hydrogen ion concentration of about 10^{-8} did not obtain at this point. The addition of 4 drops of concentrated NH_4OH was found to produce the proper color, so the technique was modified to this extent. Denis added this amount of NH_4OH in the case of trichloroacetic acid filtrates but did not mention it in precipitation from serum. Preliminary experiments had indicated that the addition of too much ammonium phosphate gave too high readings. The same was true in case of an excess of NH_4OH . After the addition of the ammonium phosphate-ammonium hydroxide mixture the tube was well scratched and allowed to stand overnight.

Confinement under constant standard conditions generally tended to decrease the plasma content of magnesium. This was possibly incidental to diminished activity but it is also possible that repeated loss of blood may have had some influence on the concentration of this substance, since there is some evidence in one of Kerr's tables that the restoration of the magnesium concentration after hemorrhage takes place more slowly than in case of the other inorganic constituents. Inorganic phosphorus concentrations varied considerably among different animals and in the

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TABLE I.

The results are expressed in mg. per 100 cc. of plasma.

Date.	Days of experiment.	Ca	Inorganic P.	Na	K	Mg	Remarks.
Dog 86.							
Mar. 25	1	11.10	7.45		11.36	2.20	
" 29	5	11.20	4.91	362	18.03	2.00	
Apr. 12	19		6.84	339	16.26		
" 17	24	10.65	7.62	350	14.56	1.90	
" 22	29			310			Operation.
" 24	31						Found dead from tetany.
Dog 87.							
Mar. 25	1	10.80	4.42		14.00	2.00	
" 29	5	12.55	6.04	354	15.55	2.30	
Apr. 12	19		4.49	356	17.80		
" 17	24		4.82	299	20.87	2.09	
" 24	31	13.91	5.42	340	17.90	1.45	Operation after bleeding.
" 26	33	12.50		331		1.61	
May 2	39	5.30	5.93	328	19.17	2.00	First observed tetany.
" 3	40						Died in tetany.
Dog 88.							
Mar. 25	1	10.55	3.55	324	12.99	2.06	
" 29	5	9.62	4.01	368	11.86	2.30	
Apr. 12	19		4.79	333	16.00		
" 17	24		4.54	310	19.17		
" 24	31	13.85		337	15.16	1.54	
" 29	36			333			Operation.
May 2	39	10.00			17.00	1.80	
" 7	44	6.00	6.33	326	15.00	1.78	Tetany. 1 cc. parathormone.
" 9	46	9.00	4.94	344	17.40	1.68	
" 21	58		6.75	314	12.64	1.25	
" 24	61	9.00	6.40	319	14.99	1.28	
" 29	66	5.45	6.06	290	13.42	1.28	Tetany. 1 cc. parathormone.
June 3	71	Found dead, position did not indicate tetany.					
Dog 92.							
May 9	1	11.00	3.63	350	16.61	1.75	
" 21	13	12.40	4.44	314	17.96	1.60	
" 24	16	13.00	5.33	331	16.64	1.43	
" 27	19						Operation.
" 29	21	8.10	5.93	314	9.68	1.55	
" 31	23	7.75	9.41	320	15.34	1.64	Tetany. 1 cc. parathormone.
June 3	26	5.10	5.20	315	14.20	1.50	Tetany.

TABLE I—*Concluded.*

Date.	Days of experiment.	Ca	Inorganic P.	Na	K	Mg	Remarks.
Dog 93.							
May 9	1	11.00	4.44	365	15.70	1.80	
" 21	13	13.00	4.82	354	16.36	1.45	
" 24	16	14.55	5.00	320	16.97	1.50	
" 27	19						Operation.
" 29	21	6.25	6.96	312	11.99	1.37	Tetany. 2 cc. parathormone.
" 31	23	10.05	7.73	332	14.38	1.49	
June 3	26	8.38	5.46	320	11.72	1.49	
" 4	27	6.35	6.84	319	18.53	1.54	Tetany. 1 cc. parathormone.
" 6	29						Dead.

	Dog 87.		Dog 88.		Dog 92.		Dog 93.	
	Na	K	Na	K	Na	K	Na	K
Average before operation.....	339	17.16	334	15.03	332	16.73	346	16.34
" after "	330	19.17	318	14.69	316	14.77	321	14.54

same animal at different times, with no apparent correlation with any other factor. Sodium was generally decreased slightly with confinement. Potassium concentrations varied more in normal dogs than did any other salt except phosphorus, but the general levels were practically constant. Magnesium ranged between 1.45 and 2.20 mg. per 100 cc.; sodium between 300 and 370 mg.; figures were obtained for potassium as low as 11.36 mg. per 100 cc., while the upper limit was seldom more than 20 mg.

In all, over 150 determinations were made, usually in duplicate, often in triplicate. Fairly satisfactory checks were obtained in all determinations. Those for potassium were less satisfactory than any of the others. In spite of the inadequacy of the methods for sodium, potassium, and magnesium, it is believed that the results are reliable, at least for comparative purposes.

In order to conserve space, only a few protocols are tabulated. Table I is composed of the condensed results of the observations on five dogs which are, we believe, fairly representative of the entire series. It happens that in all of these calcemia was higher

after several days of confinement, although our general experience has been quite the opposite. That these variations were not due to changes in the reagents is proved, not only by the frequent check determinations, but also by the fact that on the same day simultaneous determinations were often made on animals in various stages of confinement and also on operated dogs. It is probable that the high phosphatemia in Dog 86 before operation bears some relation to the early death of this animal, illustrating again the importance of this substance in the production of tetany.

Sodium concentrations tended to decrease slightly with confinement on this diet. When the averages of the figures obtained before operation are compared with those of the postoperative period, it is found that the latter are always slightly lower. In case of Dog 88, the final figure shows that there was a reduction of greater absolute and comparative magnitude than was found under any other circumstances. It is difficult to determine whether the postoperative reduction is but a continued expression of the preoperative trend or an actual parathyroid deficiency influence. Very probably the first suggestion is the correct one, although it must be admitted that no similar reduction in sodium concentration occurred in any normal animal when confined for periods much longer than in this one case. However, there is certainly no relation between changes in sodium concentration and the occurrence of tetany. Hastings and Murray (1) found no difference in the concentration just before and 2 days after operation in the one dog for which a complete record was obtained.

The general level of potassium concentrations was fairly constant before operation. If the variations noted are of significance at all, the general trend is toward an increase. Except in three cases, there was a sharp decrease for 2 or 3 days after operation, with a general tendency toward restoration later. The variations noted are in no way to be correlated with the occurrence of tetany. In two animals the average of the postoperative concentrations of potassium (exclusive of the first determination, which was usually low) was slightly but definitely lower than the preoperative average; in all other cases, when a sufficiently large number of postoperative observations was made to permit of statistical average, there was no significant difference. Hastings and Murray (1) found a slight decrease 2 days after operation in one animal,

Salvesen, Hastings, and McIntosh (2) found no significant variations in either sodium or potassium in animals in tetany after administration of phosphates.

Magnesium concentrations showed no significant variations at any time, aside from the general downward trend during confinement. Salvesen, Hastings, and McIntosh (2) invariably found a decreased concentration of magnesium when dogs were in tetany, after ingestion of phosphates, but in this series, there was certainly no constant reduction at the time of tetany.

Observations on calcium and phosphorus were generally in accord with previous findings (Reed, Lackey, and Payte (11)). There was a progressive fall in calcium and usually a tendency to increased concentration of inorganic phosphorus. It is true that in a few of our normal animals there was an occasional pronounced increase of short duration. This was also noted in previous investigations. However, the general level, when the survival period was sufficiently long to permit of comparison, was higher than in the normal. Greenwald (12) and his collaborators have repeatedly called attention to the decreased excretion of phosphorus in parathyroidectomized animals and have emphasized that this decreased excretion is not always indicated by an increased concentration in the blood. As previously reported (11), whenever it could be established definitely that the blood sample was drawn just before, or in the early stages of the first attack of tetany, the inorganic phosphorus concentration was, with few exceptions, approximately equal to, or greater than, the calcium concentration, giving a Ca:P ratio of approximately 1. In subsequent attacks, this relationship was less constant, although in our experience this ratio was approximately maintained during attacks of tetany for periods varying from a week to 5 weeks. It should be mentioned also that this ratio is more likely to occur if blood is drawn in the early stages of an attack than if drawn in the later stages or just after the attack. Discrepancies between our results and those of Greenwald are possibly explainable on this basis. This point was more fully discussed in an earlier paper (11) and several citations were made to similar results reported by others. After the acute stage was past, this ratio could be maintained for some days without the occurrence of an attack of tetany. In only two of the eight dogs surviving to this stage was there any permanent restoration of the normal proportions of

calcium and phosphorus. Therefore, it appears that the production of latent tetany does not invariably depend on the stimulation of accessory parathyroid tissue, as has been claimed. When calcium continues low and phosphorus high for several days, and the animal continues in good health and free from tetany, it is obvious that some mechanism of adaptation is involved that is not clearly understood. It is clear, so far as our results show, that this adaptation does not directly involve the plasma concentration of any of the constituents included in this study except calcium and phosphorus, and these only to a minor degree. It is possible that carbohydrate metabolism is involved, as suggested in another paper (Reed (13)).

SUMMARY.

Simultaneous determinations of calcium, inorganic phosphorus, sodium, potassium, and magnesium in the plasma of sixteen dogs, both before and after thyroparathyroidectomy, over long periods, showed that the only significant changes that could be associated with the occurrence of tetany were to be found in the concentrations of calcium and phosphorus. The production of latent tetany does not necessarily depend on the stimulation of accessory parathyroid tissue, since this condition may readily occur without restoration of calcium and phosphorus to normal concentrations.

Normal concentrations of these five constituents in the blood of dogs confined to a stock diet are reported.

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THE EFFECT OF THE CURING PROCESS UPON THE VITAMIN A AND D CONTENT OF ALFALFA.*

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1. Vitamin A Studies.

Early in the history of vitamin studies, alfalfa (1-3) was recognized as an important source of vitamin A. In view of the extensive use of dried alfalfa as a roughage for domestic animals, it was of interest to determine whether curing processes affected the vitamin A content. The samples investigated were dried on the ground, as in the usual field curing process, and by a mechanical drying process.

Curing of Samples.

Both samples were taken from the same portion of a field of alfalfa on the same day. The cutting was the second of the season, July 31, 1928.

Sample A.—This sample was cut at 3 p.m. and taken from the field at 5 p.m. It was spread out on a lawn at 6 p.m. and allowed to remain there, consecutively, through 4 nights and 3½ days. The 1st day was rainy and partly cloudy but the remaining days were hot and sunshiny. At the end of this period the hay was brownish green in color, much of the green color having disappeared. It would be classed as a poorly cured hay, as far as greenness is concerned, but it was typical of the alfalfa that is obtained in regions where cloudy and rainy weather often prevails during the haying season or under conditions of haying management whereby the greater part of the green color is lost even during clear weather.

* Journal Series paper of the New Jersey Agricultural Experiment Station, Department of Agricultural Biochemistry.

Sample B.—This was cut at 1 p.m. and left on the field in windrows until 3 p.m., when it was taken to the drier. The drying was done by the Mason process (4) at the Walker-Gordon Farms, Plainsboro, New Jersey. With this particular machine, the period of drying was 30 to 35 minutes and the temperature of the heated air as it passed from the furnace to the drying tunnel was 127–129°. The dried product was as green as the fresh material but the shade of green was a little darker than that of the undried material.

Experimental Procedure.

The procedure for the determination of vitamin A was essentially that outlined by Sherman and Munsell (5) except where otherwise indicated. The breeding colony ration was the Sherman Diet 13, consisting of 2 parts of ground whole wheat, 1 part of whole milk powder, and 2 per cent of the weight of the wheat as sodium chloride (6), modified by the incorporation of 10 per cent meat scrap (Swift's) at the proportionate expense of the wheat and milk. Young white rats, weaned at 28 days of age and weighing between 35 and 55 gm., were placed upon a basal, vitamin A-free diet, which was essentially that described by Sherman and Munsell, Diet 380, but modified to include the antirachitic factor. It was composed of purified casein 20 per cent, Osborne and Mendel (7) salt mixture 4 per cent, sodium chloride 1 per cent, dried yeast 10 per cent, corn-starch 63 per cent, and 2 per cent of olive oil, containing 25 mg. cholesterol per cc., the oil and dissolved cholesterol having been irradiated with a quartz mercury vapor lamp for 30 minutes at 60 cm. The vitamin B complex was determined in the yeast and if sufficiently potent the amount was decreased to 5 per cent and the corn-starch increased correspondingly.

During the pretest period the animals were kept in groups but during the test period they were confined in individual cages. After transfer to the individual cages, yeast and the olive oil solution of cholesterol were removed from the basal diet and fed as separate supplements, 300 to 500 mg. of the former, depending upon its potency, and 5 drops of the latter per day (except Sunday). Corn-starch replaced these two ingredients in the basal diet. All animals used in the test groups were represented by litter mates in the control group. Not more than one animal from a litter

was used in a test group and in six cases litter mates appeared in each test group. In so far as possible an equal distribution of sexes was made.

The depletion period averaged 5 to 6 weeks. An individual was ready for the test period when growth had definitely ceased and no gain was made in 3 to 5 days, and it was considered desirable to have some evidence of ophthalmia. In practically every case an eye disturbance was observed which varied from the whitening of the edge of the lid and loss of the usual beady appearance to a complete closure of the eye with marked reddening. The ophthalmias were cured during the 1st week of the test period, in the case of Samples A and B.

Preparation and Feeding of Samples.

On account of the dropping off of the leaves which results from the handling of the dried plants, a more accurate comparison of the vitamin A content can be made by comparing the leaves and stems separately. The present study is concerned with leaves only, but later studies are to be made of the stems. The leaves were picked from the stems, or sorted from those which had fallen off, and ground to a fine powder preparatory to feeding. The material was fed as a separate supplement, daily, except Sundays. Preliminary trials indicated that the mechanically dried product was 6 or 7 times as potent as that cured on the field. The daily supplement of Sample A was 150 mg. and of Sample B, 20 mg. Difficulty was experienced in inducing the animals to eat the dry, powdered leaves, but they consumed the material readily when the olive oil supplement, noted above, was added to it.

Results.

The average gain in weight in the 8 week test period was practically the same for both test groups (Table I), but the amount of the increase was more than the 25 gm. gain in 8 weeks recommended by Sherman and Munsell (5). The coefficient of variation, 24 per cent, however, for Sample B is practically the same as that reported by Sherman and Burtis (8), for an 8 week test period in which an average gain of 25.89 gm. was obtained, with a larger number of animals. In the case of Sample A, the variability is higher, the coefficient being 30 per cent. Although the

delicacy of the test may not be as great as when there is an average gain closer to that recommended by Sherman and Munsell, the results indicate that the vitamin A potency of the machine-dried material is at least 7 times that representing a field sample, cured so that the greater part of the green color is lost.

Attention has been called in a number of instances to the association of vitamin A potency and greenness in plant tissue. Thus Coward (9) has noted the loss of vitamin A when a leaf dries up and dies, and Dye, Medlock, and Crist (10), in a paper which summarizes studies of this relationship, have shown an association between greenness in lettuce leaf tissue and vitamin A potency. Also Steenbock, Hart, and their associates (11) found that alfalfa

TABLE I.
Summary of Rat Growth Records. Vitamin A in Alfalfa.

	No. of animals	Average weight at beginning of test period.	Average weight at end of test period.	Average gain in 8 wk. test period.	Coefficient of variation
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>
Sample A, field-cured, 150 mg.	12	97	145	48	30
Sample B, machine- dried, 20 mg.	12	88	138	50	24
Controls, no supple- ment.	15	95	Average weight at death, 79 gm., average survival period, 13 days.		

which was completely bleached by long exposure to sunlight lost its vitamin A potency. The findings in the case of alfalfa confirm these observations in that the fine powder obtained by grinding the leaves of the field-cured sample was brownish green in color, as compared with the distinctly green color of the machine-dried sample. In a number of other tests with alfalfa, of a qualitative nature, made in this laboratory, the same relationship between color and vitamin A potency was found to hold. Dutcher (12) in a review article remarks that: "Practical feeders have observed that bright green alfalfa, properly dried and cured, is superior to the light colored crops, bleached out by unsatisfactory climatic conditions or careless agricultural practice."

2. Vitamin D Studies.

Steenbock, Hart, and their associates (11) have demonstrated that, "The antirachitic properties of hays are related to their exposure to sunlight." Clover hay, for instance, made in the sunlight showed considerable calcifying power, whereas a sample of the hay made in the dark was inactive, the white rat being used as the test animal. They also pointed out that the antirachitic potency of clover hay which had been excessively weathered was reduced as compared with hays less exposed to dew and rain. Steenbock and Hart had noted as early as 1913 (13) that the feeding of green grasses to goats and keeping them out of doors greatly improved the assimilation of lime as compared with keeping the animals indoors on a ration of straws and grains.

In view of these findings the problem naturally presented itself as to the antirachitic value of alfalfa hay dried artificially by the Mason process (4), mentioned above, a few hours after cutting, as compared with that of a hay cured in the sun.

Curing of Samples.

The samples were collected at the same time and from the same portion of the field as those described for the vitamin A studies.

Sample C.—This sample was cut at 3 p.m. and taken from the field at 3.30 p.m. It was transported to the laboratory and spread on the floor overnight. On the following morning it was exposed to weak sunshine for 1½ hours and then taken indoors on account of rain. During each of the following 2 days, it was spread on canvas in bright sunshine from 9.30 a.m. until 4.30 p.m. At other times it was kept indoors, out of sunlight. The total exposure to direct sunlight, after cutting, was about 16 hours.

Sample D.—The handling of this sample was the same as that of Sample C, except that it was allowed to dry on the laboratory floor in diffused daylight, but not in direct sunlight.

Sample B.—This sample was obtained by the same process as Sample B of the vitamin A studies.

Experimental Procedure.

The procedure for the assay of vitamin D has been described previously (14) and is based on that outlined by Bills, Honeywell, and MacNair (15). The diet used for the production of rickets

was the Steenbock Ration 2965, a high calcium-low phosphorus ration (16). Young rats, weaned at 24 days, developed rickets in 20 to 22 days. After a 5 day test period, the degree of cure produced by the material under examination was estimated by the Shipley line test (17).

Preparation and Feeding of Samples.

As in the case of the vitamin A studies, only the leaves were used, in order to obtain a more accurate comparison. They were ground to a fine powder and incorporated in the rickets-producing diet, at the expense of the yellow corn, so that they constituted 15 per cent of the diet. Sample D was irradiated with a Cooper Hewitt Uviarc (poultry treater) quartz mercury lamp, operated on 110 volts, A.C. The exposure was for $\frac{1}{2}$ hour at 60 cm.

The question might be raised as to the disturbance of the calcium-phosphorus ratio in the rickets-producing diet by the introduction of alfalfa. Table II shows the calcium-phosphorus ratio of the Steenbock Ration 2965 and of this diet after the incorporation of 15 per cent of each of the alfalfa samples. The test diets are still high calcium-low phosphorus in character and the variation from the rickets-producing diet is probably not greater than would be found among diets prepared from different lots of yellow corn and of wheat gluten. Furthermore, the tests of the three samples are comparable in that the ratios of the test diets are practically the same.

Results.

Table III is a summary of the rat records. Details of food consumption and growth are not given for each individual but an animal's record was rejected if the food consumption was less than 2 gm. for any one day of the test period or if the average consumption was less than 4 gm. per day. Also a loss of weight during the test period resulted in the discarding of an animal.

The readings obtained in the case of Sample C, sun-dried, are higher than those of Samples D and B, air-dried in the absence of sunlight and machine-dried, respectively. In the case of the latter two samples, the degree of the cure is slightly greater in the case of Sample D, but the difference between the two samples is not considered significant.

To determine whether the material exposed to sunlight had reached its maximum antirachitic potency, a portion of Sample D,

TABLE II.
Calcium-Phosphorus Ratios of the Rickets-Producing and Test Diets.

Diet.	Ca:P
Steenbock Ration 2965:	4.9:1
Sample C incorporated	5.6:1
" D "	5.6:1
" B "	5.3:1

TABLE III.
Summary of Line Test Readings. Vitamin D Determinations.

	No. of animals.	Average weight at beginning of test period.	Average weight at end of test period.	Average gain in 5 day test period.	Average daily food consumption in 5 day test period.	Line test readings.	Estimated average reading.
		gm.	gm.	gm.	gm.		
Sample C, sun-dried.	11	70.0	75.4	5.4	7.7	3+, 2+, 2+, 2+, 2+, +, +, +, -, -, -	1+
Sample D, air-dried in absence of sunlight.	14	64.3	68.6	4.3	7.3	2+, +, +, +, +, +, +, +, +, -, -, -, -, -	<1+
Sample B, machine-dried.	9	69.0	75.0	6.0	7.0	+, +, +, -, -, -, -, -, -, -	<1+
Sample F, irradiated portion of Sample D.	11	69.0	75.0	6.0	6.0	3+, 3+, 3+, 2+, 2+, 2+, 2+, 2+, 2+, 2+, 2+.	2+
Controls, at beginning of test period.	10					All negative.	

air-dried in the absence of sunlight, was exposed to the radiations of a quartz mercury lamp, as described above. That still more activatable substance was present is evident from the higher

degree of curing which resulted in the case of the irradiated sample, referred to as Sample F in Table III. Although the method for the determination of the antirachitic factor used in the present investigation is not directly comparable with that employed by Steenbock, Hart, and their associates (11) in their studies of clover hay, the results in the cases of Samples C, D, and F are in general agreement.

The results of the vitamin A studies described in the first part of the paper would lead to the question of the vitamin A content of Sample C, which lost the greater part of its green color during the sun-drying process.

In Table IV is recorded the result of the feeding of a 50 mg. supplement of leaves of Sample C. The result is not directly

TABLE IV.
Vitamin A in Sample C.

Supplement.	No. of animals.	Average weight at beginning of test period.	Average weight at end of test period.	Average gain in 8 wk test period.	Coefficient of variation
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>
Sample C sun-cured, 50 mg.	8	108	128	20	46
Controls.	Same as in Table I.				

comparable with that of Sample B, Table I, because an equal gain was not made in the 8 week period, but a loss of vitamin A has taken place owing to the process of curing. Also the eye disturbance persisted for a longer time than in the case of Sample B. Hence the increase in vitamin D potency, obtained by drying in the sun, was accompanied by a decrease in vitamin A.

SUMMARY.

1. Alfalfa leaves from plants dried by artificial heat, by the Mason process, were found to contain at least 7 times as much vitamin A as the leaves from hay that was cured in the field so that the greater part of its green color was lost.

2. The sample which contained the larger amount of vitamin A was green as compared with the brownish green color of the field-cured sample.

3. The leaves of the artificially cured plants contained only a small amount of the antirachitic vitamin.

4. When the alfalfa was dried in the sun, without exposure to dew or rain, there was an increase in the antirachitic potency of the leaves, but it was accompanied by a decrease in vitamin A content.

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A NEW METHOD FOR DETERMINING THE ACTIVITY OF CERTAIN OXIDASES, WITH A PRELIMINARY STUDY OF THE POTATO OXIDASE.

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In connection with some electrometric determinations of the pH of blood with a quinhydrone electrode a very slow drift toward a more positive potential was noted, especially when small amounts of the quinhydrone were employed. This was accelerated by bubbling air through the liquid. That the change in potential was not due to a change in blood reaction was indicated by the fact that making the liquid more concentrated in quinhydrone tended to restore the original potential. This partial restoration of potential seemed to be due to a partial restoration of the 1:1 ratio of quinone to hydroquinone, and thus the drift appeared to be due to oxidation of the hydroquinone in the quinhydrone to quinone. It was thought that observations of the change of potential of such an electrode might offer a very simple method for detecting the presence of certain sufficiently active oxidases or oxygenases, for comparing the activities of preparations of these enzymes and, perhaps, for studying such catalyzed oxidation reactions themselves.

The principle underlying such a method would depend on the fact that the potential of a reversible system consisting of any substance and its oxidation product is determined, other factors remaining constant, by the ratio of the concentrations of material in the oxidized state to that in the reduced state. The substance quinhydrone happens to furnish such a reversible oxidation-reduction system since it dissolves giving equimolar concentrations of quinone, the oxidized form, and hydroquinone, the reduced

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form. Thus, if other factors such as pH, reference electrode, etc., are kept constant, any oxidation of hydroquinone can be followed by attending the potential rise, inasmuch as such oxidation would alter the original 1:1 concentration ratio of quinone to hydroquinone, which obtains in quinhydrone, to some higher value.

With this in mind the indicative experiments were made which are reported in this paper. An ordinary potentiometer set-up was employed, with a saturated calomel half-cell as reference electrode.

The potato oxidase was selected as most suitable. This has been reported to effect the oxidation of catechol to quinone (1, 2), and might be expected to cause rapid oxidation of hydroquinone. The optimum pH reported for the oxidation of catechol was 6.4. In this work the pH maintained was about 5.8 to 6. Potatoes were ground and macerated with acetate-acetic acid mixture about 0.4 molal in total acetate. The liquor was roughly squeezed out through cheese-cloth by hand. It loses activity fairly rapidly when kept open to the air, but when kept under nitrogen it can be used for a good many hours without great loss in activity.

The general procedure was as follows: Into small beakers a volume of potato extract and sufficient buffer to make 30 cc. were introduced. To this was added a known amount of quinhydrone and the initial potential reading taken. Then, at a given time, a constant vigorous stream of air was bubbled through the liquid so as to keep the entire solution agitated, and the time required for a rise through a certain potential, usually 30 millivolts, noted. Such a rise would correspond to a change in the quinone-hydroquinone ratio from 1:1 to 10:1. In order to make sure that this change in ratio was responsible for the rise in potential, the air current was removed at the end of this 30 millivolt rise and a second measured quantity of quinhydrone added. From the known amounts of quinhydrone and the known ratio change it is easy to calculate the theoretical final potential and compare this with the value obtained experimentally. An example will make this procedure clear. We have

$$E = E_0 + \frac{RT}{nF} \ln \frac{(\text{quinone})}{(\text{hydroquinone})} = E_0 + 0.0295 \log \frac{(\text{quinone})}{(\text{hydroquinone})}$$

since $n = 2$ for this reaction. The symbol \ln stands for the natural logarithm, while \log stands for the common logarithm. Thus for a 1:1 ratio of quinone to hydroquinone $E = E_0$, the magnitude of which depends on the pH, the particular reference electrode, etc., and in these experiments would be the initial reading. Here one is not primarily interested in the value of this magnitude but rather in the differences in potential as oxidation proceeds. For the quinone-hydroquinone ratio 10:1, $E = E_0 + 0.0295$, corresponding roughly to a rise of 30 millivolts.

The quinhydrone was added by pipetting known volumes of a solution made by dissolving 0.5 gm. in 200 cc. of distilled water. Suppose that 2 cc. of such a solution had been originally added and then, after the 30 millivolt rise in potential, 5 cc. additional be added. The first quantity may be thought of as 1 part of quinone and 1 part of hydroquinone, which is transformed during the oxidation to 1.82 parts of quinone and 0.18 part of hydroquinone; *i.e.*, a ratio 10:1. The addition of 5 cc. of quinhydrone to such a solution would amount, on the same basis, to the addition of 2.5 parts of quinone and 2.5 parts of hydroquinone and would bring the final ratio to 4.32 parts of quinone to 2.68 parts of hydroquinone or a value of 1.612. This gives

$$E = E_0 + 0.0295 \log 1.612 = E_0 + 0.0061$$

I.e., the potential should be restored in such a case from 30 millivolts above the initial value to 6.1 millivolts above it.

Effect on Potential of Bubbling Air through Acetate-Acetic Acid Mixture.

In the absence of potato extract an hour's bubbling of air produced no rise in the potential of the quinhydrone. As a matter of fact a slight fall was noted of from 0.5 to 1.5 millivolts which may have been due to volatilization of acetic acid from the mixture. None of the experiments described required nearly this length of time.

Effect on Potential of Bubbling Nitrogen through the Oxidase Solution.

Table I shows the effect of bubbling nitrogen gas through two samples of potato extract.

TABLE I.
Effect of Bubbling Nitrogen through Oxidase Solution.

Sample No.	Extract volume.	Volume of quinhydrone solution.	Potential.						
			0 min.	5 min.	10 min.	15 min.	20 min.	25 min.	30 min.
	cc.	cc.	mv.	mv.	mv.	mv.	mv.	mv.	mv.
1	25 0	2 0	112 0	111 5	111.5	111.5			
2	30.0	2 0	119 0	118.5	118 5	118 5	118 5	118 5	118 5

At the end of the time intervals noted the nitrogen gas stream was removed and the air stream introduced. The potentials in both cases began at once to rise, Sample 1 requiring 8 minutes and Sample 2 requiring 6.25 minutes for a 30 millivolt rise.

TABLE II.
Effect of Enzyme Concentration on Time of Quinhydrone Oxidation.

Extract volume.	Min. required for rise of:			Original E.M.F.	Measured E.M.F., 5 cc. quinhydrone added.	Calculated final E.M.F.
	10 mv.	20 mv.	30 mv.			
cc.				mv.	mv.	mv.
Run 1.						
10.0	11.00	25.0	49 50	122 0	128 0	128 1
15 0	5 50	10 0	21 50	123 0	128 5	129 1
30.0	1.75	4 0	6 25	121 0	127.0	127.1
Run 2.						
10.0	11.00	27.0	52 0	122 0	127 5	128 1
15.0	6 00	12 0	24 0	121 0	127 0	127 1
20.0	3.67	9 0	16 0	121 0	127.0	127.1
30 0*	1 67	4.0	6 0	120.0	126.0	126 1

* It was immediately after this determination that another 30 cc. sample (Sample 2, Table I) was run, nitrogen gas being bubbled through for 30 minutes. When the air was turned on, the times for the above potential rises were, respectively, 10 mv., 1.67 minutes; 20 mv., 4 minutes; 30 mv., 6.25 minutes. These results duplicate the sample given in Table I very closely. Times are exact to about 5 seconds.

Effect of Concentration of Enzyme on Time of Oxidation of Hydroquinone.

Varying volumes of potato extract of the same sample were diluted to 30 cc. and then 2 cc. of quinhydrone solution added. Results of two representative runs are given in Table II. They

were made on the same sample kept under nitrogen and were run 4 hours apart. After each determination 5 cc. of quin-

TABLE III.

Relation between Time and Quantity of Hydroquinone Oxidized When Ratio of Enzyme to Substrate Is Kept Constant and Concentration of Substrate Varied.

Extract volume.	Quin-hydrone solution.	Min. required for rise of:			Original E.M.F.	Added volume of quin-hydrone.	Final measured E.M.F.	Calculated final E.M.F.
		10 mv.	20 mv.	30 mv.				
cc.	cc.				mv.	cc.	mv.	mv.
Run 1.								
30.0	4.0	3.25	6.5	9.33	100.0	10.0	106.0	106.1
15.0	2.0	3.25	6.5	9.50	100.0	5.0	106.0	106.1
7.5	1.0	4.00	7.0	11.00	100.0	5.0	103.5	103.8
Run 2.								
30.0	4.50	4.75						
20.0	3.00	6.00						
15.0	2.25	6.00						
10.0	1.50	6.75						
Run 3.								
30.0	3.0	1.50	2.50	3.20	104.0	5.0	112.0	112.1
20.0	2.0	1.75	2.50	3.20	104.0	5.0	110.5	110.1
15.0	1.5	1.50	2.33	2.75	104.0	5.0	109.0	108.9
10.0	1.0	1.00	2.00	2.75	104.0	5.0	108.0	107.8
Run 4.								
30.0	6.0	3.50	5.50	6.75	104.0	6.0	115.0	115.1
20.0	4.0	2.75	4.66	6.20	104.0	5.0	115.0	113.7
15.0	3.0	2.50	4.66	6.20	104.0	5.0	113.0	112.1
10.0	2.0	3.00	5.70	8.00	104.0	5.0	111.0	111.1
Run 5.								
30.0	3.0	3.67	9.5					
20.0	2.0	3.67	11.0					
15.0	1.5	3.50	11.0					
10.0	1.0	2.75	9.0					

hydrone solution were added and the resulting potential compared with the theoretical value calculated as shown above. The E.M.F. measurements were made only to the nearest 0.5 millivolt.

If the reaction takes place in a single step these data indicate that it is of a higher order.

It was thought that perhaps simpler numerical relationships might be obtained by keeping the initial ratio of enzyme to quinhydrone constant. Table III gives data from some experiments of this kind.

Finally, Table IV gives data on the relationship between time and quantity of hydroquinone oxidized in the presence of a constant amount of enzyme.

TABLE IV.

Relation between Time and Quantity of Hydroquinone Oxidized in Presence of Constant Amount of Enzyme When Concentration of Substrate Is Varied.

Extract volume.	Quinhydrone solution	Min. required for rise of:			Original E.M.F.	Added volume of quinhydrone.	Final measured E.M.F.	Calculated final E.M.F.
		10 mv.	20 mv.	30 mv.				
cc.	cc.				mv.	cc.	mv.	mv.
Run 1.								
10.0	1 0	1 00	1 75	2 50	104.0	5 0	108 0	107 8
	2.0	1 75	3 33	4 75	106 0	5.0	112 0	112.1
	3.0	2 75	5 33	7 50	105.0	5 0	113 5	113.1
	5 0	5.25	11.00	15 00	105 0	5 0	117 0	116.1
Run 2.								
15.0	1 5	1 75	2.25	2 67	105 0	5 0	110 0	109.9
	3 0	2.00	3 33	5 50	105 0	5.0	114 0	113.1
	6.0	3 50	6.75	10.75	105 0	6 0	117.0	116.1
Run 3.								
20 0	2.0	1.75	2 50	3 20	104 0	5 0	110 0	110.1
	6.0	4.50	6 50	9 10	104 0	6.0	116.0	115.1

DISCUSSION.

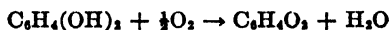
The presence of oxidase has ordinarily been detected and its activity measured either by measuring the rate of disappearance of gaseous oxygen (3), or by the formation of a colored oxidation product from a colorless reduced reagent (4, 5). The method proposed here is, where applicable, easier than the first mentioned and has the advantage over the second that color or turbidity does not interfere with its use.

In 1917 Reed (6) suggested that the measurement of the oxida-

tion potential might be of importance in the study of oxidases. To the authors' knowledge no other work directly bearing along this line has appeared, and even in the article by Reed no mention was made of any reversible reference system for determining or fixing any definite potential.

It is not proposed at this time to attempt to discuss the mechanism of the reaction, but merely to point out that, while the times of oxidation through the same potential change are not proportional to the enzyme concentrations (Table II), nevertheless there is a rough proportionality, through the range of conditions studied, between time of oxidation and quantity of quinhydrone substrate (7) (Table IV), and also if a constant ratio of enzyme to substrate is maintained, the times of oxidation through a given potential change are, while not equal, at least of the same order. Through the range studied Schütz's rule does not seem to hold, but it may be found to be more closely approximated with more concentrated substrates and for a smaller potential change. There seems to be a rapid slowing down of the oxidation for concentrations of enzyme somewhat smaller than those reported.

It should be borne in mind that most methods of studying enzymes are based on measurements of activity rather than concentration, and that the two magnitudes may be by no means proportional (7). Another point should be considered especially in the study of reversible systems such as that of quinone-hydroquinone. Unlike fermentation reactions as ordinarily carried out where, after the CO_2 has reached a fairly definite pressure it is removed from the reaction and a constant pressure maintained, here the oxidized component accumulates and builds up an increasing oxidation potential against which the reaction must be forced. Thus the conditions at the end of a 30 millivolt rise are by no means the same as at the initial potential. This can be exemplified by a consideration of one of the factors; namely, the partial pressure of the oxygen gas. Ostensibly this is kept constant at about 0.2 atmosphere if air be used as a source. If we assume the reaction



then, for the same concentration of hydroquinone at an oxidation potential 30 millivolts above some standard potential, we would

find the *apparent* oxygen pressure so far as the oxidation reaction is concerned is much smaller than 0.2 atmosphere.

Thus, for an oxygen cell with two oxygen electrodes at different partial pressures, p_2 and p_1 ,

$$E = \frac{RT}{nF} \ln \frac{p_2}{p_1} = 0.0148 \log \frac{p_2}{p_1}$$

since, for O_2 , $n = 4$. A difference of 30 millivolts, then, for the same oxidizing power of gaseous oxygen would require a higher partial pressure of oxygen gas calculated from the above relation:

$$0.030 = 0.0148 \log \frac{p_2}{p_1}$$

where $\log \frac{p_2}{p_1} = 2$, or $\frac{p_2}{p_1} = 100$.

This means that a pressure of 100 times 0.2 or about 20 atmospheres would be necessary to maintain the same oxidizing tendency as is obtained initially by the oxygen in ordinary air.

We hope in the future to study some of these factors experimentally, using the method described here, not only extending the limits of various concentration factors, but poisoning the solution at various oxidation potentials.

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CREATINE METABOLISM IN A CASE OF GENERALIZED MYOSITIS FIBROSA.*

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Myositis fibrosa has been defined by Steiner (1) as "a single or multiple inflammation of the muscles, mostly subacute or chronic, which generally begins in the lower extremities, and presents but slight constitutional symptoms. Eventually the muscle tissue concerned is largely or wholly replaced by connective tissue, and quite pronounced muscle atrophy may then be observed." A sand-bag feel or a wood-like hardness of the muscles is said to be characteristic of the disease.

The generalized form of myositis fibrosa is exceedingly rare, as may be judged by the fact that despite the relative certainty with which it may be diagnosed, only a few cases are to be found in the literature. Since the first authentic description by Gies in 1879 (2), single cases have been reported by each of the following clinicians: Kreiss (3), Janicke (4), Gowers (5), and Batten (6). A more recently reported case is that of Burton, Gowan, and Miller (7). To these may perhaps be added the case briefly described by Hoover (8), the diagnosis for which must remain somewhat unsettled, since it was not confirmed by histological study of the tissues nor by postmortem examination. Finally, under "dermatomyositis," Hoover describes three cases, one of which (Case 3), presents many features which are not altogether typical of dermatomyositis, but which resemble more nearly the symptoms associated with myositis fibrosa.

Relatively little is known of the pathology of this rare condition, and as to its etiology no definite opinion has yet been advanced. A search of the literature has failed to reveal a single reference to a biochemical study of this obscure disease.

A detailed account of the clinical observations and pathological findings will be reported elsewhere. The present paper will be limited principally to a consideration of the creatine metabolism,

* Part of the data contained in this communication was presented before The Thirteenth International Physiological Congress at Boston.

and only such portions of the history will be given as may have a direct bearing on the questions involved.

The patient, a negro boy nearly 15 years of age, was admitted to the hospital complaining of stiffness of the whole body and pain in the chest. About 1 year previously, his mother had noticed that he was not as active as formerly and that he seemed to move about slowly and deliberately. However, he continued to go to school and to do odd jobs around the house. A few weeks later, the patient noticed a peculiar stiffness in his hands which made it somewhat difficult for him to perform the finer movements. This stiffness became slowly but progressively worse. 4 or 5 weeks after the recognition of the stiffness of his hands, he noticed a similar condition beginning in his legs, which seemed largely to involve the knees, and which interfered to some extent with walking. In September, 7 months after the beginning of his trouble, he started to school as usual, but was unable to play with other boys as he had previously done. While doing calisthenics at school, he noticed that he was not able to bend over as far as the other pupils and that his back seemed to be getting stiff and rigid. During this period of his illness his appetite remained good. As far as he could tell, he had lost no weight. At no time during his illness did the patient complain of any pain in his muscles or joints, a feature which is said to be characteristic of all but the initial stages of generalized myositis fibrosa.

On physical examination of the muscular system, all the muscles of the body, some more than others, seemed to be indurated and hard, and to have lost their normal elasticity, resulting in limitation of movement. To the palpating hand, they imparted the sensation of being firmer and stiffer than normal, and not unlike a sand-bag. The volumes of the muscles were fairly well preserved with the exception of the deltoid and pectoral muscles which showed considerable atrophy. None of the muscles was painful, either on palpation or movement.

A few days after admission to the hospital, the patient began to show an occasional slight afternoon rise in temperature. This disappeared during the month of February, but in March the afternoon temperature became more or less continuous, reaching on one occasion as high as 102°F. Examination of the chest showed signs of infiltration in both apices. x-Ray studies con-

firmed the clinical findings and suggested that the process was in all probability tuberculous in nature. There was no cough or expectoration and sufficient sputum for examination could not be obtained.

From the standpoint of creatine metabolism, several points which have been brought out in the foregoing excerpts from the patient's history, such as the age, the occasional afternoon rise in temperature, and, possibly, the complicating tuberculosis, will require special consideration.

Relation of Meat Intake to the Elimination of Creatine and Creatinine.—On the routine hospital diet which included a small amount of meat once a day, the patient eliminated, in the course of 24 hours, 1.146 gm. of creatine and creatinine, of which 0.672 gm. was present as creatinine and 0.474 gm. as creatine. For the next 3 days meat was excluded from the diet, which consisted of eggs, oatmeal, cream, butter, bread, cabbage, and other vegetables. On this diet there was little change in the elimination of creatine and creatinine. When the amount of meat was somewhat increased, as on February 13, the creatine output was also increased. The effect of a higher intake of meat was determined on analyzing the urine of February 26. For several days previously, as well as on the day of the urine collection, the patient was given liberal amounts of meat. Although the total nitrogen amounted to only 17.07 gm., an increase of 7.07 gm. over the preceding period, the output of creatine and creatinine was doubled, the increase in creatine being 0.99 gm. (expressed as creatinine) or 78 per cent of the extra "total creatinine" elimination.

As is indicated by the data in Table I, the patient exhibited a marked creatinuria even on a meat-free diet, and the ingestion of a moderate amount of meat produced a very marked increase in the creatine output. That the patient had not lost the ability to convert a portion of the exogenous creatine to creatinine is manifested by the fact that after being on the high meat diet for several days, the creatinine output was considerably higher than on previous experimental days.

During this period of observation the patient was afebrile and his weight remained fairly constant at 49 ± 1 kilo.

The question to be considered in this connection is whether to attribute the creatinuria entirely to the muscular condition, or

whether the age of the patient is a factor to be taken into account. Unfortunately, the data for the creatine elimination of normal boys for the age period of the patient are both meager and conflicting. In his recent monograph, Hunter (9) has tabulated the results of a number of investigators for the purpose of providing information of the quantities of creatine which children of different ages may be expected to excrete on varying intakes of protein, but on what are usually considered creatine-free diets. Not a single analysis is given for boys older than 9 to 9½ years, the analyses for this age being based on the work of Harding and Gaebler (10) who found the creatine elimination, on a nitrogen intake of approximately 15 gm., to be 14.7 per cent of the "total creatinine" in one of the boys, and 6.1 per cent in the other.

In his important paper which established the presence of creatinuria in normal children, Rose (11) gives analyses of specimens of urine of two boys, aged, respectively, 14 and 15 years. The diets were not controlled and it was impractical in these cases to collect 24 hour specimens. The creatine elimination of the younger boy was 27.9 per cent of the total, and of the older boy, 25.3 per cent. Rose observed no creatinuria in boys above this age. Folin and Denis (12) examined a specimen of urine of a 17 year old boy, who was a vegetarian, and found 18 mg. of creatine in 100 cc. On the other hand, Krause (13) was unable to find creatine in the urine of boys after the age of 7, his data including analyses of the urine of male subjects up to the age of 16. More recently, Cameron and Gibson (14) observed no creatinuria in two boys, aged 9 and 12 years. Obviously, there is need for more information regarding the creatine metabolism of boys at the age of puberty and during adolescence.

In this connection it is to be stated that the patient was very well developed sexually, giving the appearance of a much older boy.

Although this evidence was taken into account, it is felt, nevertheless, that it would be safer not to exclude the factor of age as possibly contributing to the creatinuria in the case under discussion.

In considering the data in Table I, the effect of the tuberculosis may probably be ruled out. During the period of these observations, the patient's temperature was normal and not subject to

wide fluctuations. The symptoms of the disease were not sufficiently marked in order to make a positive diagnosis. Nevertheless, the complication is not to be ignored even at this stage of the disease if the various factors are to be brought in relation to each other. For this reason, the effect of tuberculosis on creatine metabolism may be briefly considered. In so far as the disease is associated with periodic elevation of temperature, an increased creatinine elimination and even some creatinuria may be expected. Meyer (15) studied the metabolism of two female patients (ages not stated) with advanced tuberculosis and found creatinuria in both. Van Hoogenhuyze and Verploegh (16) found creatinuria in a case of chronic pulmonary tuberculosis (male, age 72), but in this case the patient exhibited a high temperature. Of much greater value in appraising the data obtained in the present study is the information furnished by McClure (17). In a case of pulmonary tuberculosis (male, age 19), creatinuria was absent as a rule. Of twenty-nine 24 hour specimens of urine analyzed, creatine in very small amounts was found in only three. This patient exhibited approximately the same fluctuations in temperature as those observed in our patient during March. McClure's patient, though maintained on a modified Shaffer-Coleman (18) diet, showed considerable variation in the daily output of creatinine, whereas in our case the "total creatinine" elimination was fairly constant, both on the house diet and the meat-free diet.

In a second case of pulmonary tuberculosis reported by McClure (male, age 27), small amounts of creatine were present in only four 24 hour specimens and absent in twenty-seven, in spite of the fact that the patient was febrile, the temperature frequently rising above 102°F.

Effect of Feeding Creatine.—On March 8, the patient received 1 gm. of creatine hydrate (0.758 gm. of creatinine), all of which was recovered in the urine collected during that 24 hour period, 92.1 per cent being excreted as creatine and the remainder as creatinine. It should be mentioned that the creatine preparation itself was practically free from creatinine.

On the following day 2 gm. of creatine hydrate were administered, of which 64 per cent was recovered that day. Of the amount thus recovered, 91.8 per cent was present as creatine and 8.2 per cent as creatinine. The urine collected during the subsequent

24 hour period contained extra creatine and creatinine to account for an additional 27 per cent, but in this case, 54.9 per cent was present as creatine and 45.1 per cent as creatinine. All the calculations in this, as well as in the preceding experiment, are based on the analyses of March 3, which have been taken for control.

On March 11, 5 gm. of creatine hydrate were given in 1 gm. doses at half hourly intervals. The extra "total creatinine" the 1st day was 2.657 gm., or 70.1 per cent, nearly all of which (94.05 per cent) was present as creatine. The extra elimination on the 2nd day accounted for 14.5 per cent and on the 3rd day, 7.13 per cent, making a total recovery of 91.73 per cent. The proportion present as preformed creatinine was 5.95 per cent on the 1st day, 18.2 per cent on the 2nd day, and 48.2 per cent on the 3rd day. The significance of these results will be discussed presently. In the next 24 hour period the creatine and creatinine elimination returned to approximately the normal values for this individual, as shown by the data in Table II.

The preceding experiments were controlled by giving the patient 2 gm. of creatinine. Of this amount, 90.5 per cent was recovered the 1st day (*i.e.*, actually within $15\frac{1}{2}$ hours), and an additional 6 per cent was present in the urine of the following day.

For the next few days the patient was given the usual house diet, without the meat. To provide the usual amount of protein, two eggs were given at each meal. While on this diet, the patient was given 1 gm. of creatine hydrate at 9.30 a.m., on March 20. On the basis of the calculations on the control analyses of March 17 to 18, 77.85 per cent of this was recovered within the next $20\frac{1}{2}$ hours. Sufficient extra creatine and creatinine was found in the urine of the subsequent 24 hours to account for an additional 10.55 per cent. Thus, 88.4 per cent of the creatine was recovered. If the calculations were based on the analysis of the 24 hour specimen of March 19, an even higher value for the total recovery would be obtained.

Powis and Raper (19) have observed that on giving creatine to a normal child in the morning, most of it was excreted during the ensuing 12 hours, but if the same amount was given before going to bed, a large proportion was retained. To determine whether creatine retention could be obtained by administering it at night, 1 gm. was given at 8.30 p.m., shortly before the patient's

TABLE II.
Fate of Creatine and Creatinine.

Date.	Maximum and minimum temperature.	Volume of urine in 24 hrs.	pH	Total N.	Urea N.	NH ₄ N	Uric acid N.	Undetermined N.	Total creatinine	Preformed creatinine.	Creatine (expressed as creatinine).	Per cent of total creatinine excreted as creatine.	Extra total creatinine.	Extra creatinine.	Extra creatine (expressed as creatinine).
Mar., 1929	°F.	cc.		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
3	97.8 97.4	2090	6.7	8.59			0.105		1.28	0.70	0.58	45.6			
8	99.8 98	2040	6.8	11.77					2.04	0.76	1.28	62.7	0.76	0.06	0.70
9	99.8 99.2	1820	6.0	11.48	9.38	0.50	0.214	0.55	2.25	0.78	1.47	65.2	0.97	0.08	0.89
10	100.6 100.2	885	5.6	11.45	9.23	0.57	0.192	0.83	1.69	0.885	0.805	47.6	0.41	0.185	0.225

House diet.

" " 1 gm. creatine hydrate (0.758 gm. creatinine). Total recovered, 100%; 7.9% was creatinine, 92.1% creatine. House diet; 2 gm. creatine hydrate (1.516 gm. creatinine). Recovered 64%; of which 8.2% was creatinine, 91.8% creatine.

House diet. Recovered 27%; of which 45.1% was creatinine, 54.9% creatine. Total recovery within 48 hrs., 91%.

TABLE II—Concluded.

Date.	Maximum and minimum temperature.	Volume of urine in 24 hrs.	pH	Total N.	Urea N.	NH ₄ N	Uric acid N.	Undetermined N.	Total creatinine.	Preformed creatinine.	Creatinine (expressed as creatinine).	Per cent of total creatinine excreted as creatinine.	Extra total creatinine.	Extra creatinine.	Extra creatinine (expressed as creatinine).	
Mar., 1929	°F.	cc.		gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	
20	99.4 97.8	1640	6.0	10 107	780.58	0.16	0.90	1.82	0 72	1 10	60.40	59	0 06	0 53	0 53	Meat-free diet; 1 gm. creatine hydrate (0.758 gm. creatinine) given at 9.30 a.m. Total recovered 1st day, 77.85%; of which 89.8% was creatine, 10.2% creatinine. Analyses of Mar. 17-18 taken for control.
21	99.8 98.4	1770	5.9	10 238.20	530 15	0 86	1.31	0 71	0 60	45 80	0 08	0 05	0 03	0 03	0 03	Meat-free diet. Recovered 2nd day, 10 55%; of which 62.5% was creatinine, 37.5% creatine. Analyses of Mar. 17-18 taken for control. Total recovery, 88.4%.

22	99.4 98.4	1510	6.0	9.30	7.06	0.55	0.16	0.97	1.51	0.67	0.84	55.60	28	0.01	0.27	Meat-free diet; 1 gm. creatine hydrate given at 8.30 p.m. Recovered 1st day, i.e., within 9.5 hrs., 37%; of which 3.6% was creatinine, 96.4% creatine.
23	102 98.4	1750	6.0	11.12	8.97	0.69	0.13	0.72	1.63	0.68	0.95	58.30	40	0.02	0.38	Meat-free diet. Recovered 52.8% 2nd day; of which 5% was creatinine, 95% creatine. Total recovery for 2 days, 89.8%.
24	100.4 98	2230	6.0	9.49	7.33	0.70	0.16	0.83	1.27	0.635	0.635	50.0				Meat-free diet.
26	99.8 99.2	650	5.8	6.55	4.55	0.45	0.15	0.91	1.33	0.65	0.68	51.1				" " 400 gm. glucose. Test for sugar in urine faintly positive.
27	99 98.4	705	6.2	8.87	6.00	0.47		2.01	6.0	601	1.415	70.00	746	-0.034	0.78	High meat diet. Patient ate about 300 gm. of ground beef in morning, but was too ill to continue experiment. Analysis of Mar. 24 taken for control.
28	98.6 97.4	650	6.0	8.65	6.12	0.61		1.78	0.45	1.33	74.70	51		-0.185	0.695	Meat-free diet. Patient ill.

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bedtime. In the urine voided at 6 a.m. the following morning, sufficient extra creatine and creatinine were found to account for 37 per cent of that administered. Most of the remainder was recovered in the urine of the following day. The total recovery, in this experiment, was 89.8 per cent. Apparently, the retention was no better when the creatine was given at night than when given in the morning.

On the following day the composition of the urine returned to the normal for this patient. The creatine elimination was now 50 per cent of the "total creatinine." To determine whether carbohydrate ingestion might have some effect on the creatinuria, 400 gm. of pure glucose were given on March 26, in addition to the meat-free diet. Although by this time the condition of the patient had become worse, the sugar was exceedingly well tolerated, the urine for that day giving but a faint Benedict's test for reducing substance. Presumably, the metabolism of the carbohydrate had no effect on the creatine metabolism, unless the somewhat increased elimination of both creatine and creatinine may be taken to indicate a slight stimulation of tissue catabolism. It may be further stated that at no time during these observations did the patient exhibit any symptoms of acidosis.

On the following day it was intended to determine again the effect of a high meat intake. In the morning the patient slowly ate about 300 gm. of ground beef, but during the rest of the day he was too ill to continue the experiment. Although the total nitrogen elimination for that 24 hour period was only 8.87 gm., the "total creatinine" was 2.016 gm., an increase of 0.746 gm. over the control period of March 24. The creatinuria continued to be very marked on the following day.

The inability of the patient to store creatine is of fundamental significance, the results of our observations standing out in striking contrast to those that have been made in normal adults by Folin (20), Myers and Fine (21), Rose and Dimmitt (22), Chanutin (23), and others. Even in children, whose ability to retain exogenous creatine is much less than in adults, the administration of amounts of creatine, comparable to those taken by the patient, results in the recovery of much smaller quantities in the urine. It is only in very young children and in infants that complete recovery has been occasionally reported (Powis and Raper (19),

Beumer (24), and Gamble and Goldschmidt (25)). In the experiments of Gamble and Goldschmidt, of 88 mg. of creatine (anhydrous) fed to a 10 months old infant, weighing 7.2 kilos, 31 mg., or 35 per cent, were recovered in the urine of the next 2 days. When 0.264 gm. was given, 51 per cent was recovered in 2 days. In these experiments the infant was on a low protein diet. In a subsequent experiment, the infant was placed on a high protein diet, in the form of whole milk, and given 0.264 gm. of creatine. All of this was recovered in the urine of the ensuing 5 days. Apparently, even the 10 months old subject of these experiments was, as a rule, better able to retain creatine in his tissues than our 15 year old patient with generalized myositis.

Practically complete recovery of exogenous creatine has been observed by Powis and Raper (19) in a case of amyotonia congenita in a child of 4, and by Gibson and Martin (26) in a case of pseudohypertrophic dystrophy in a woman.

The deficiency of creatine retention in our patient seems to be very marked only when compared with the relatively prolonged type of creatine storage which may be assumed to occur on feeding creatine to normal adults. However from the standpoint of a transient type of storage, an appreciable retention of creatine in our subject seems to be indicated. The mere fact that all of the absorbed creatine was not at once excreted in the urine is in itself evidence that it was taken up to some extent by the tissues. Additional support for this idea is to be found in the fact that the administration of 5 gm. of creatine had no effect on the creatine content of the blood. This result, as indicated in Table III, differed from that obtained on feeding 2 gm. of creatinine, an excretory product, for a marked elevation of creatinine in the blood was promptly observed. But of more significance in supporting the idea of a transitory storage of some of the creatine are the deductions which may be drawn from the data in Table II. It will be observed that after feeding 2 gm. of creatine, nearly all of the extra "total creatinine" determined on the 1st day was in the form of creatine. However, on the 2nd day, the amount present as creatinine was appreciable, in fact, 45.1 per cent of the total. The results were similar when 5 gm. were given, the amount of extra creatinine being 5.95 per cent of the total on the 1st day, 18.2 per cent on the 2nd, and 48.2 per cent on the 3rd. There

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can be little question of the origin of this extra creatinine, especially if one recalls Benedict and Osterberg's experiments with dogs (27), as well as those of Chanutin (23), and Rose, Ellis, and Helming (28) on man, which show (1) that an increase of creatinine does not immediately follow creatine feeding, but apparently occurs after the body has retained a proportion of the creatine fed, and (2) that whereas, on withholding creatine after periods of prolonged administration, the creatinuria markedly diminishes or disappears at once, the increased elimination of creatinine persists for some time.

TABLE III.
Summary of Blood Analyses.

Figures are expressed in mg. per 100 cc. of blood.

		Non-protein N.	Uric acid.	Creatinine.	Creatine.	Glucose.	
<i>1929</i>							
Feb.	7	28.6	5.6			91	House diet.
"	10	28.6	5.6	1.3	3.7		Meat-free diet.
"	13	30	5.7	1.15	3.85		Moderate amount of meat.
"	26	30	5.1	1.13	3.85	90	High meat diet.
Mar.	11	27	5.3	1.07	3.73		Blood taken at 4.30 p.m., 30 min. after last dose of creatine. Total given, 5 gm. creatine hydrate.
"	15	28	5.4	2.5	4.00		Blood taken 30 min. after 2 gm. dose of creatinine.

It may even be questioned, though, because of the complexity of the relations involved, not entirely on the basis of our own observations, whether the muscles in this case of myositis have actually lost much of their capacity to convert creatine into creatinine. The justification for this question, here, lies in the conclusions reached by Benedict and Osterberg (27) that the formation of creatinine from creatine is a definitely limited process, that the conversion is not a *direct* one, but that one or more intermediate reactions are probably involved, that the conversion occurs slowly, and that even on prolonged administration of creatine (to dogs) only about one-third of the retained creatine is converted to creatinine, the remainder following different metabolic paths. What-

ever these intermediate reactions may be, it seems likely that they depend on the retention of the creatine in the tissues in some form not easily liberated. We are therefore inclined to the view that even in this generalized muscular disease, if exogenous creatine is retained, a proportion, perhaps the physiologically normal proportion, is changed to creatinine. We believe that we are justified in assuming that the derangement of the creatine metabolism in this condition, and possibly in other diseases of the muscular system, is due not to failure to convert creatine into creatinine, *per se*,

TABLE IV.

Creatine Content of Muscles and Its Relation to Anatomical Changes.

Muscle.	Creatine in 100 gm.	Degen- eration.	Fibro- sis.	Inflam- mation.	Crea- tine.
	mg.				
Soleus	324	1	2	1	1
Quadriceps femoris.....	313	2	6	2	2
Iliacus.....	160	3	6	9	8
Intercostal.....	172	4	3	6	7
Diaphragm.....	159	5	1	3	9
Psoas.....	204	6	5	4	5
Rectus abdominis.....	217	7	6	5	4
Sartorius.....	229	8	4	7	3
Deltoid.....	197	9	6	8	6

Least degeneration, fibrosis, or inflammation is denoted by a grade of 1. Most degeneration and inflammation are indicated by a grade of 9. Since several muscles showed approximately the same degree of fibrosis, the grade 5 signifies maximum fibrosis in this series. Intermediate degrees of anatomical change are graded accordingly. In the last column grade 1 denotes the most, and grade 9 the least, amount of creatine.

but primarily to an inability to "fix" the creatine in a form in which it would be retained in the tissues for more than just a very brief time.

Muscle Creatine.—The only extensive series of determinations of creatine (and creatinine) in human muscle is that of Denis (29). In a group of five normal individuals, she obtained values ranging between 360 and 421 mg. Within this range fall the values obtained by Myers and Fine (30) and Shaffer (31). In autopsy material obtained from individuals dying of acute diseases, Denis found the creatine content of the muscle to be normal, although in

a few instances low values were observed. The muscle creatine in a proportion of the chronic diseases was found to be considerably below the normal range, but in nearly all instances in which this was the case, the clinical histories showed that the patients had been ill for many months and were for some time before death in an extremely cachectic and emaciated condition. To this class belonged patients with Graves' disease, gastric and intestinal carcinoma, etc. Since our patient definitely did not belong to this category of chronic, cachectic diseases, the low creatine content of his muscles (Table IV) must be associated with their diseased condition.

Toward the end of March, the patient's condition became rapidly worse. On March 29, a biopsy was performed to secure muscle (quadriceps femoris) for histological study. A portion of this was analyzed for creatine by the procedure of Ochoa and Valdecasas (32) the determinations being made in triplicate, and 100 mg. of muscle used for each analysis. Death occurred on the morning of March 30. At autopsy, performed the same morning, several specimens of muscle were obtained and analyzed for creatinine. We have since had an opportunity of comparing the method of Ochoa and Valdecasas with that of Rose, Helmer, and Chanutin (33) and are assured of its reliability especially when at least 100 mg. of tissue are used in each determination.

Owing to the limited number of analyses of normal human muscle, comparison of the data in Table IV with the normal is not entirely possible except in the case of the psoas, the muscle which Denis selected for analysis in her series of determinations. However, from the data for the creatine content of other human muscles, that are to be found in the literature, it is clear that the values for the various muscles, listed in Table IV, are very low, the creatine content in some instances being less than one-half that occurring normally.

Microscopic examination revealed as much as 30 per cent degeneration of some of the muscles (deltoid, sartorius). By careful comparison with each other the various muscles were graded according to the amount of normal tissue still present. Arranged according to the normal amount of muscle still present, the order was: soleus, quadriceps femoris, iliacus, intercostal, diaphragm, psoas, rectus abdominis, sartorius, and deltoid. The soleus

showed most normal muscle, the deltoid, least. Arranged according to the amount of fibrosis, the order was: deltoid, rectus abdominis, iliacus, and quadriceps femoris equally fibrosed; next in order the psoas, sartorius, intercostal, soleus, and diaphragm. The diaphragm showed least fibrosis. Arranged in the order of inflammation present, the order was: iliacus, deltoid, sartorius, intercostal, rectus abdominis, psoas, diaphragm, quadriceps femoris, and soleus, the last showing the least amount of inflammation. While these classifications are to be regarded only as fair approximations, they are nevertheless not without significance, especially when compared with the results of the creatine determinations. It is to be mentioned that these gradations were made without knowledge of the chemical findings.

In Table IV, a grade of 1 denotes either least degeneration (most normal muscle), fibrosis, or inflammation. Most degeneration, as well as most marked inflammation, is represented by 9. Since several muscles showed approximately the same amount of fibrosis, the grade 5 indicates maximum fibrosis in this series (deltoid, rectus abdominis, iliacus, and quadriceps femoris). The parallelism between the amount of anatomical change, particularly between the degree of the inflammatory process, and the creatine content, while in no sense absolute, is nevertheless very striking. Thus, the soleus, which appeared most normal and showed least inflammation, contained more creatine than any of the muscles. The quadriceps femoris (obtained at biopsy), while it exhibited a considerable amount of fibrosis, showed relatively little of other degenerative changes, and in harmony with this, the creatine content was also relatively high. On the other hand, the iliacus and deltoid which showed a great deal of inflammation and other pathological changes, were very low as regards creatine content.

The myocardium contained 159 mg. per 100 gm., a value slightly below that given by Constabel (34) for heart muscle. Histological examination revealed a slight enlargement of the fibers, apparently a hypertrophic change. The muscle cytoplasm was more granular than normal and there was a slight increase of fibrous tissue, as well as a few scattered mononuclear inflammatory cells. The data for the diaphragm show most departure from this parallelism. Whether this may be due to a low creatine content of this muscle, normally, remains to be determined.

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Purine Metabolism.—The uric acid content of the blood was consistently above normal and seemed to be uninfluenced by the increased white blood cell count which occurred in March (the white blood cell count in February was 10,000; in March, 15,000). However, the uric acid elimination increased appreciably during March. These changes may have been wholly a manifestation of the tuberculous involvement, an inference which seems warranted from Wells' discussion (35) of purine metabolism in tuberculosis.

SUMMARY.

The results are presented of a metabolism study of a case of generalized myositis fibrosa, in the earlier stages, in a 15 year old boy.

Marked creatinuria was a constant feature, even on a creatine-free diet, the amount of creatine eliminated being more than 40 per cent of the "total creatinine."

The patient manifested an almost complete inability to retain, for more than very short periods, exogenous creatine. However, of the fraction which found transitory storage in the tissues, a portion was apparently converted into creatinine.

Analyses of the muscles revealed the fact that they were abnormally low in creatine content.

A relationship of the creatine content of the various muscles to the severity of the anatomical changes particularly to the degree of inflammation, has been demonstrated. It appears that an inflammatory condition of the muscles interferes with the normal storage of creatine.

We wish to acknowledge the cooperation of Dr. Titus H. Harris of the Department of Neurology and Psychiatry, who made the original diagnosis, and of Dr. Henry C. Hartman of the Department of Pathology, who confirmed the pathological findings.

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THE CHEMISTRY OF THE LIPOIDS OF TUBERCLE BACILLI.

VII. ANALYSIS OF THE SOFT WAX FROM TUBERCLE BACILLI.*

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INTRODUCTION.

The crude wax obtained from the chloroform extract of the human type of tubercle bacillus, Strain H-37, yielded on purification, as described in a former paper (1), two fractions; viz., a white solid substance designated as purified wax and a soft yellowish salve-like mass which was called soft wax. The present report deals with the analysis of the latter fraction. The work is far from complete and several constituents have not been identified. Our attention has been chiefly focused upon the nature of the fatty acids that were liberated on saponification, but incidentally some facts regarding other constituents have been observed and recorded.

The water-soluble constituents, after saponification, contained only traces of reducing sugars and only a small amount of a syrupy substance was isolated, which probably consisted of glycerol since it gave a distinct acrolein reaction when heated with acid potassium sulfate.

The ether-soluble components were found to consist of a small amount of a neutral perfume-like substance, a small quantity of unsaponifiable wax similar to that obtained from the purified wax (1), and a large percentage of true fatty acids.

The fatty acids were separated by means of the lead soap-ether method according to the Gusserow-Varrentrapp (2, 3) pro-

* The present report is a part of a cooperative investigation on tuberculosis and it has been supported partly by funds provided by the Research Committee of the National Tuberculosis Association.

cedure into solid and liquid acids. The small amount of solid saturated acids thus obtained was apparently a mixture composed principally of palmitic and stearic acids. The liquid acids had an iodine number of 33.5 but after catalytic reduction with hydrogen and platinum oxide (4) only a very small amount of the reduced saturated acid could be isolated. It is probable, therefore, that very highly unsaturated fatty acids are present. Moreover, it was of interest to find that the reduced acid was not homogeneous but could be separated by crystallization from acetone into two fractions. The top fraction melted at 80–82°, while the more soluble portion of the reduced acid had approximately the melting point of stearic acid. This observation would indicate that at least two unsaturated fatty acids are present in this lipid fraction.

What we regard as a matter of particular interest was the isolation of a liquid saturated optically active fatty acid of high molecular weight which was obtained from the ether-soluble lead soap after the catalytic reduction of the unsaturated acids. The substance is analogous to phthioic acid (5) and it is present in relatively large amount, constituting the principal fatty acid in the soft wax. It was obtained as a faintly yellow oil which solidified when cooled and then melted at 22.5°. When the acid was allowed to stand for a longer time at room temperature a portion of it separated in semicrystalline aggregates. It is probable, therefore, that it represents a mixture of tuberculostearic acid and phthioic acid similar to that obtained from the acetone-soluble fat (6). This fraction of the liquid saturated acids has been studied in Dr. Sabin's laboratory and it has been found to possess biological properties analogous to those of phthioic acid.

As judged by the cleavage products obtained in this analysis it would seem as if the soft wax corresponds in composition more nearly to a complex glyceride than to a wax. In fact the only wax-like substance observed either in the purified wax or in the fraction at present under examination is the material designated as unsaponifiable wax. The unsaponifiable wax possesses, however, peculiar properties. It contains apparently at least one free carboxyl group since it has acid properties and forms salts. It must also contain at least one free hydroxyl group because it forms an acetyl derivative. Further work will be necessary before one can

determine whether the substance is a wax or a more highly complex body.

All of the lipid fractions isolated from the human type of tubercle bacilli, Strain H-37, have now been subjected to a preliminary analysis and the cleavage products have been determined. All of these fractions have been found to contain very similar fatty acids which may be classified under three groups: (a) solid saturated fatty acids such as palmitic, stearic, and cerotic acids, (b) liquid unsaturated acids such as oleic and linoleic acids, (c) liquid saturated optically active acids such as tuberculostearic and phthioic acids.

From a chemical point of view the outstanding interest in these studies is the isolation of a series of new saturated fatty acids of high molecular weight which are either liquid at room temperature or are low melting solids and which are optically active. The optically active acid has been named phthioic acid and it possesses important biological properties. It has been shown by the experiments conducted by Drs. Sabin, Doan, and Forkner¹ at the Rockefeller Institute that the biological activity of the lipoids of tubercle bacilli can be duplicated by the phthioic acid alone. The substance is therefore regarded as a maturation factor for monocytes and epithelioid cells.

EXPERIMENTAL.

The soft wax used in these experiments had been isolated from the mother liquor after purification of the crude tubercle bacillus wax as described in a former paper (1) and the material had been kept in an atmosphere of carbon dioxide until it was analyzed. The substance formed a yellowish brown salve-like mass at room temperature and it possessed a slight but agreeable perfume-like odor.

On analysis it was found to contain merely a trace of nitrogen and only 0.08 per cent of phosphorus. The phosphorus content would indicate a very slight contamination of phosphatides. When tested by the Hanus method, the iodine number was 32.1.

¹ The results of these biological studies will be published shortly by these investigators.

Hydrolysis by Means of Alcohol and Hydrochloric Acid.²

In order to determine the nature and amount of the water-soluble constituents a preliminary hydrolysis of the soft wax was carried out as follows: 25 gm. of material, dissolved in a little ether, were refluxed with 350 cc. of alcohol containing 20 cc. of concentrated hydrochloric acid.

The material liquefied in the hot alcohol without dissolving and the color gradually darkened. After refluxing for 13 hours the mixture was allowed to cool, when the oily substance solidified, forming a wax-like cake of dark brown color. About one-half of the alcohol was distilled off and the residue was diluted with water and extracted with ether. The ethereal solution was washed with water, filtered, and evaporated to dryness. The residue, after drying in a vacuum desiccator, formed a dark brown soft solid which weighed 24.5 gm.

Examination of the Aqueous Solution.

The aqueous solution and the washings mentioned above were combined, concentrated, and examined for water-soluble constituents. An aliquot was dried, when a syrupy residue was obtained which was equivalent to about 1 gm. of solids in the total solution. The solution contained a trace of reducing sugar and very small amounts of phosphoric and glycerophosphoric acids. The solution was evaporated to dryness *in vacuo* and the bulk of the hydrochloric acid was removed by evaporating several times with the addition of absolute alcohol. The residue was dissolved in water and neutralized with barium hydroxide. The barium phosphate was filtered off and the barium glycerophosphate was precipitated in the filtrate by adding alcohol and removed by centrifuging. The solution was then concentrated to dryness *in vacuo* and the residue was extracted with absolute alcohol. The alcoholic solution was evaporated to dryness, when a small amount of a syrupy residue was obtained. When some of this syrup was heated with acid potassium sulfate the odor of acrolein was noticeable, indicating the probable presence of glycerol.

The analytical data detailed above show clearly that the acid-

² During the hydrolysis and throughout these experiments air was displaced as far as possible by the use of carbon dioxide.

alcohol hydrolysis of the soft wax resulted in the formation of very small amounts of water-soluble constituents and that reducing sugars were practically absent. The small quantities of phosphoric and glycerophosphoric acids that were detected were probably derived, as already suggested, from a slight contamination of phosphatide. As judged by the acrolein test the principal water-soluble component was apparently glycerol. These facts show that the composition of the soft wax is decidedly different from that of the previously analyzed purified wax.

Saponification of Fat Recovered after Acid-Alcohol Hydrolysis.

The fat which was recovered after the acid-alcohol hydrolysis, 24.5 gm., was saponified by refluxing for 3 hours with 250 cc. of 5 per cent alcoholic potassium hydroxide. The material did not dissolve completely but some oily drops remained suspended in the alkaline solution and the color turned very dark. About one-half of the alcohol was distilled off and the solution was diluted with 400 cc. of water, when the oily material dissolved. The alkaline solution was extracted several times with ether, when severe emulsions formed which retained nearly all of the coloring matter. The ethereal extract was treated as will be described later.

Examination of the Alkaline Aqueous Solution.

The aqueous solution was acidified with hydrochloric acid and extracted with ether. The ethereal solution was washed with water, filtered, and evaporated to dryness. The dark brown residue which remained weighed only about 0.1 gm. It is evident, therefore, that practically all of the ether-soluble material including fatty acids had passed into the ethereal emulsion.

The acidified aqueous solution was concentrated to dryness *in vacuo* and the residue was extracted with absolute alcohol. The alcoholic solution was evaporated to dryness and the residue was again extracted with alcohol. The filtered extract was evaporated to dryness when a small amount of a syrupy residue was obtained. A sample of the syrup was heated with acid potassium sulfate, when the odor of acrolein was produced, indicating the presence of glycerol.

Examination of the Ethereal Extract.

The ethereal solution formed persistent emulsions on shaking with water but a partial separation was effected by adding potassium hydroxide and a large quantity of water and allowing the mixture to stand for several hours. The clear ethereal layer was then separated, washed with water, filtered, and the ether was distilled. After the residue had been dried, a brown semicrystalline solid remained that possessed a pleasant perfume-like odor. The substance weighed 1.7 gm.

The emulsified bottom layer, after the separation mentioned above, was acidified with hydrochloric acid and the mixture was extracted with ether. The ethereal extract was washed with water, filtered, and the ether distilled. The residue after it had been dried *in vacuo* formed a dark brown semicrystalline solid that weighed 20.6 gm.

Separation of Unsaponifiable Wax.

The material mentioned above was dissolved in 200 cc. of hot acetone and as the solution cooled nearly colorless fine globular particles separated. The precipitate was filtered off, washed with cold acetone, and reprecipitated a second time from hot acetone by cooling. The substance was then dissolved in 30 cc. of warm ether, 50 cc. of acetone were added, and the solution was cooled in ice water, when a precipitate separated which consisted of colorless fine globular particles. After the substance had been filtered off, washed with cold acetone, and dried, it formed a white amorphous powder that weighed 3.7 gm.

The substance was readily soluble in ether, chloroform, or in benzene but it was very slightly soluble in cold alcohol or acetone. For instance the last ether-acetone mother liquors mentioned above contained only 0.3 gm. of a wax-like residue. It dissolved more readily in hot alcohol or in hot acetone, but when these solutions were allowed to cool the substance did not separate in crystalline form but only fine globular particles were observed. The substance gave no coloration whatever in the Liebermann-Burchard reaction and in chloroform solution no bromine was absorbed. It is evident, therefore, that the substance was a saturated compound and that it did not contain any sterols.

Heated in a capillary tube the substance began to sinter at 57–58° and it melted at 68–69°.

Analysis.

0.1236 gm. substance: 0.1503 gm. H₂O and 0.3660 gm. CO₂.

Found. C 80.75, H 13.60.

Titration.—0.5386 gm. of substance dissolved in 40 cc. of ether and diluted with 25 cc. of alcohol required 6.24 cc. of 0.1 N alcoholic KOH, with phenolphthalein as indicator. The neutralization value corresponds to a molecular weight of 863.

In chloroform solution the substance was optically inactive.

Acetyl Derivative.—1 gm. of the substance was refluxed for 3 hours with 50 cc. of acetic anhydride. The reaction product was isolated and precipitated from a solution in ether and acetone by cooling in ice water, when a white amorphous powder was obtained which melted at 64–65°. This product was saponified by refluxing with alcoholic potassium hydroxide, the alcohol was evaporated, and the residue was acidified with dilute phosphoric acid and then distilled, with the addition of water, until the distillate was practically neutral. The amount of volatile acid in the distillate, calculated as acetic acid, amounted to 4.23 per cent.

Although this substance is probably not homogeneous, its composition and properties resemble those of the so called unsaponifiable wax previously isolated from the purified wax.

Examination of the Fatty Acids.

The first two acetone mother liquors, after the substance described above was separated, were united and evaporated to dryness. The residue was a dark brown oil which solidified on cooling and which weighed 16.6 gm.

It was dissolved in 100 cc. of warm alcohol, neutralized with potassium hydroxide, diluted with water, and precipitated with an excess of lead acetate. The lead soaps were treated with ether and the insoluble portion was separated and washed with ether. The lead soaps were decomposed by shaking with dilute hydrochloric acid and the fatty acids were isolated in the usual manner.

The ether-soluble lead soap yielded 12.8 gm. of a yellowish brown oil consisting of liquid fatty acids, and the ether-insoluble lead soap gave 3.7 gm. of a white solid crystalline fatty acid.

Liquid Fatty Acids. Isolation of Liquid Saturated Fatty Acid Analogous to Phthioic Acid.

The iodine number of the liquid fatty acids determined by the Hanus method was 33.5. In order to remove the unsaturated fatty acid the mixture was reduced with hydrogen and platinum oxide and the reduction product was separated by repeating the lead soap-ether treatment in the same manner as described for the isolation of phthioic acid (5).

The liquid saturated fatty acid isolated from the ether-soluble lead salt after reduction was a light yellow oil. Unfortunately some of the acid was lost when a flask broke, but the portion that was saved weighed 9.8 gm. The acid was decolorized in alcoholic solution by treatment with norit. The oil solidified on cooling and melted at 22.5° . On longer standing at room temperature a portion of the substance separated in semicrystalline globular aggregates. The acid was saturated because in chloroform solution there was no visible absorption of bromine.

Titration.—0.3424 gm. of acid dissolved in 50 cc. of neutral alcohol required 8.06 cc. of 0.1 N alcoholic KOH, with phenolphthalein as indicator. Molecular weight found, 424.

Rotation.—1.1852 gm. of acid dissolved in alcohol and made up to 10 cc. gave in a 1 dm. tube a reading of $+0.576^{\circ}$ at 23° ; hence $[\alpha]_D^{23} = +4.85^{\circ}$.

Analysis.

0.1164 gm. substance: 0.1351 gm. H_2O and 0.3324 gm. CO_2 .

Found. C 77.88, H 12.98.

The acid is evidently a mixture, probably consisting mainly of tuberculostearic and phthioic acids, and is similar to the mixture obtained from the acetone-soluble fat.

When a sample of this acid was tested in Dr. Sabin's laboratory it was found to possess biological properties similar to those of phthioic acid.

Reduced Acid.

The acid contained in the ether-insoluble lead salt, after the catalytic reduction, was liberated and isolated in the usual manner. It was a colorless crystalline solid which weighed only 0.3 gm. The substance was recrystallized, five times from acetone

and once from methyl alcohol, when a few mg. of colorless crystals were obtained. The substance melted at 79–80°, solidified at 78°, and remelted at 79–80°.

The mother liquors were concentrated to dryness and the residue was dissolved in 10 cc. of methyl alcohol, treated with norit, and filtered. The solution, on cooling in ice water, deposited a small amount of show-white crystals which were filtered, washed with cold methyl alcohol, and dried *in vacuo*. This fraction of the reduced acid melted at 67–68°, solidified at 62°, and remelted at 67–68°. It is probable that this acid consisted largely of stearic acid.

While we have reduced a large number of unsaturated fatty acids isolated from various fractions of the lipoids of tubercle bacilli, this is the first time that we have ever obtained any reduced acid which possessed a higher melting point than that of stearic acid. The amount of the high melting reduction product obtained in this case was too small to permit the determination of the molecular weight or the composition. The melting point, about 80°, would indicate, however, that the substance is probably behenic acid, which would have been formed by the reduction of erucic acid.

In view of the small quantity of reduced acids that was obtained one must conclude that the original mixture of liquid acids contained very highly unsaturated fatty acids.

Solid Saturated Fatty Acids.

As has been mentioned earlier the ether-insoluble lead soap from the original mixture of fatty acids yielded 3.7 gm. of a white crystalline solid. The substance was recrystallized three times from acetone, when a snow-white product was obtained that weighed 1.4 gm. It melted at 56–57°, solidified at 54°, and remelted at 56–57°. When titrated in alcoholic solution with 0.1 N alcoholic KOH, phenolphthalein being used as indicator, the molecular weights found were 291 and 293.

A second fraction of the acid, isolated from the mother liquor, also melted at 56–57° and on titration the value found for the molecular weight was 282.

The low melting point of these fractions together with the high molecular weight would indicate that a mixture of fatty acids is

present and that one of the constituents must have a higher molecular weight than stearic acid. A complete separation and identification of the acids present would require a much larger quantity of material than is now available.

A second analysis was made of the soft wax with 45.6 gm. of material and the following cleavage products were recovered:

1. Water- and alcohol-soluble syrup which gave a positive acrolein test.....	gm. 2.5
2. Neutral perfume-like substance.....	6.2
3. Unsaponifiable wax.....	5.6
4. Crude fatty acids.....	31.7
Total material recovered.....	46.0

The soft wax in this analysis was saponified directly by refluxing with alcoholic potassium hydroxide.

The substance designated unsaponifiable wax remained as an insoluble oil in the hot saponification mixture and it solidified to a wax-like cake when the solution was cooled. The alcoholic solution was decanted and the insoluble residue in the flask was treated several times with boiling alcoholic potassium hydroxide. The insoluble material, which evidently represented a potassium salt of the unsaponifiable wax, was warmed with dilute hydrochloric acid and extracted with ether. On evaporation of the ether a residue remained which was purified by several precipitations from ether-acetone solution until a pure white amorphous powder was obtained. The substance corresponded entirely in composition and properties with the unsaponifiable wax which was isolated in the first analysis. The melting point was 68–69° and on combustion the following composition was found, C 80.36, H 13.64 per cent.

The alcoholic solution, after the unsaponifiable wax had been removed, was concentrated, diluted with water, and extracted with ether. The ethereal extract yielded on concentration a soft brown mass which possessed a very agreeable odor and which has been designated as a neutral perfume-like substance. Lack of time has prevented any examination of this material.

The alkaline solution was acidified with hydrochloric acid and the fatty acids were extracted with ether, after which the aqueous solution was concentrated to dryness under reduced pressure.

The residue was evaporated several times with absolute alcohol and finally it was extracted with alcohol. The alcoholic extract was evaporated to dryness *in vacuo* and the residue was again extracted with alcohol. The filtered extract, on evaporation to dryness *in vacuo*, left a thick dark colored syrup which weighed 2.5 gm. When some of this syrup was heated with acid potassium sulfate, the odor of acrolein became noticeable.

The crude fatty acids were separated into solid and liquid acids by means of the lead soap-ether treatment. The solid fatty acid obtained from the ether-insoluble lead soap weighed 4.7 gm. It was purified by several recrystallizations from acetone and from methyl alcohol, yielding snow-white crystals. The melting point, 56–57°, was the same as that found for the corresponding acid previously described.

The liquid fatty acids were reduced with hydrogen and platinum oxide and the reduction products were separated by means of the lead soap-ether treatment. The reduced acid weighed 0.9 gm. and by recrystallizations from acetone was separated into two fractions. The top fraction, amounting only to a few mg. of snow-white crystals, melted at 82–83°, solidified at 80°, and remelted at 81–82°. A second fraction of the reduced acid was isolated from the mother liquor which melted at 65–66°.

The liquid saturated fatty acid, analogous to phthioic acid, was isolated from the ether-soluble lead soap. It formed a light yellow thick oil which partly solidified on standing at room temperature and it weighed 21.5 gm.

Titration.—0.2560 gm. of substance dissolved in 50 cc. of neutral alcohol required 6.66 cc. of 0.1 N alcoholic KOH, with phenolphthalein as indicator. Molecular weight found, 384.

Rotation.—0.4574 gm. of substance dissolved in alcohol and made up to 10 cc. gave in a 1 dm. tube a reading of +0.155°; hence $[\alpha]_D^{20} = +3.38^\circ$.

It is evident from the data recorded above that the quantities and the properties of the cleavage products of the soft wax as determined in the second analysis are essentially similar to those described in the earlier portion of this paper.

SUMMARY.

1. The fraction of the chloroform-soluble lipoids of the human type of tubercle bacilli, Strain H-37, designated as soft wax, has been analyzed. The composition of this fraction corresponds more nearly to that of a complex glyceride than to a wax.

2. After saponification the cleavage products were separated into: (a) 5.48 per cent water- and alcohol-soluble syrup which gave a positive acrolein reaction and presumably contained glycerol, (b) 95.39 per cent ether-soluble constituents.

3. The ether-soluble material contained: (a) 13.59 per cent neutral perfume-like substance, (b) 12.28 per cent unsaponifiable wax, (c) 69.51 per cent crude fatty acids.

4. The fatty acids were separated into: (a) solid saturated fatty acids, m.p. 56–57°, presumably a mixture consisting largely of palmitic and stearic acids, (b) liquid fatty acids containing a small amount of unsaturated fatty acids and a large amount of liquid saturated fatty acids.

5. The liquid saturated fatty acid is optically active and possesses biological properties analogous to phthioic acid.

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THE CHEMISTRY OF THE LIPOIDS OF TUBERCLE BACILLI.

VIII. CONCERNING THE UNSAPONIFIABLE WAX.*

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(Received for publication, October 1, 1929.)

INTRODUCTION.

The principal ether-soluble constituent isolated after saponification of the so called purified wax obtained from the chloroform extract of the human type of tubercle bacillus, Strain H-37, was described in a former paper (1) under the designation of unsaponifiable matter or unsaponifiable wax. A review of the literature was given in that publication and will not be repeated here.

The substance that we obtained must correspond to the unsaponifiable matter or higher alcohols described by former investigators as occurring in the fat from tubercle bacilli because it was the only solid cleavage product obtained from the wax that possessed properties of a higher alcohol. In several particulars, however, our results differ from those that have been reported previously.

The material under investigation formed salts and it also reacted with acetic anhydride, thus indicating that it possessed both acid and alcoholic properties. It differed also from the substance isolated by Tamura and described under the name of mykol (2) in that it gave no definite bromine derivative. The compound appears to be very complex and to have a high molecular weight. It is very stable and can be boiled for many hours with alcohol containing hydrochloric acid or sulfuric acid or with sodium

* The present report is a part of a cooperative investigation on tuberculosis and it has been supported partly by funds provided by the Research Committee of the National Tuberculosis Association.

ethoxide without undergoing any noteworthy changes in its properties. In other words it seems to act as a unit substance which can be recovered unchanged after numerous precipitations and after other more violent reactions.

When the unsaponifiable wax is heated under reduced pressure, it boils and decomposes between 280–310°, a solid saturated crystalline fatty acid, apparently hexacosanic acid, distils over, and a non-volatile neutral substance is left which contains a higher percentage of carbon than the original material.

The great stability of the compound suggests that it must contain peculiar linkages, possibly an ether linkage such as was found by Heilbron and Owens (3) to exist in batyl and in selachyl alcohols. In fact when the unsaponifiable wax was heated with hydriodic acid, as in the usual Zeisel determination for methoxyl groups or for glycerol (4), a volatile iodide was liberated. We have not been able to identify this iodide, but if it were isopropyl iodide it would correspond to about 4 per cent of glycerol.

At the present time the unsaponifiable wax is perhaps only of academic interest. It possesses no biological activity. In studies performed in Dr. Sabin's laboratory at the Rockefeller Institute it was found that the compound gave no reaction that was comparable to that of the phosphatide or of phthioic acid.

EXPERIMENTAL.

The so called unsaponifiable matter or unsaponifiable wax which was isolated from the purified wax as described in a former paper (1) was used in these experiments. The general properties and the solubility of the substance in various solvents have already been given.

The amount of this material obtained after the purified wax had been hydrolyzed or saponified was 56 per cent. It represented, therefore, the principal ether-soluble constituent of the wax. The substance, as isolated after saponification with alcoholic potassium hydroxide, always contained ash. The residue, consisting of potassium carbonate, which was left on combustion, amounted to 4.1 to 4.2 per cent.

An ash-free substance was obtained by treating the ethereal solution of the potassium salt with hydrochloric acid and washing with water. The substance was a snow-white, dense, amorphous

powder. Heated in a capillary tube it softened at 56° and melted at 57–58°. In chloroform solution the substance showed no optical activity and it was apparently a saturated compound because when dissolved in chloroform it did not decolorize a dilute solution of bromine.

There was no loss in weight on drying at 61° *in vacuo* over dehydrite.

Analysis.

0.1654 gm., 0.1180 gm. substance: 0.2014 gm., 0.1441 gm. H₂O and 0.4960, 0.3532 gm. CO₂.

Found. C 81.78, 81.63, H 13.62, 13.66.

Another preparation was analyzed with the following results: C 81.64, H 13.56.

Various purified preparations were titrated in hot alcoholic solutions with 0.1 N alcoholic KOH, with phenolphthalein as indicator, when the following values were obtained.

0.7732 gm. substance:	5.61 cc. 0.1 N KOH.	Mol. wt. 1378.
0.5261 " " "	: 3.88 " 0.1 " " "	1353.
0.8156 " " "	: 5.85 " 0.1 " " "	1394.
0.8695 " " "	: 6.30 " 0.1 " " "	1380.

Bromine Derivative.

The substance, 1 gm., was dissolved in 50 cc. of ether and 10 cc. of 5 per cent bromine in glacial acetic acid were added. There was no immediate evidence of absorption of bromine. After the solution had stood for 16 hours at room temperature, it still contained a large excess of bromine. The solution was diluted with glacial acetic acid until it turned cloudy, and it was then cooled in a freezing mixture. A white amorphous precipitate separated on cooling which was filtered off and washed with methyl alcohol. The substance was reprecipitated from ether-glacial acetic acid solution by cooling, filtered, washed with methyl alcohol, and dried *in vacuo* over sulfuric acid and potassium hydroxide. The product was a snow-white amorphous powder which weighed 1 gm. and gave a qualitative reaction for halogen. The substance melted at 58–59°.

Analysis.—(Heating with CaO.)

0.2498 gm. substance: 0.0126 gm. AgBr.

Found. Br 2.14.

The small amount of bromine contained in this substance was probably due to substitution and evidently no definite bromine derivative is formed.

Potassium Salt.

The substance, 0.7732 gm., was dissolved in 500 cc. of hot alcohol and titrated with 0.1 N alcoholic KOH, with phenolphthalein as indicator. Required, 5.61 cc. of 0.1 N KOH. The neutralization value corresponds to a molecular weight of 1378.

The alcoholic solution was cooled in ice water, when a snow-white precipitate separated. The material was not crystalline but consisted of fine globular particles. The precipitate was collected on a small Buchner funnel, washed with methyl alcohol, and dried *in vacuo*. The snow-white amorphous powder weighed 0.75 gm. The substance had no sharp melting point but began to soften at 60° and formed an opaque melt at 64°, which became transparent at about 90°.

For analysis the substance was burned at a low heat in a platinum crucible.

0.2434 gm. substance: 0.0104 gm. K_2CO_3

Found. K_2CO_3 4.27.

Silver Salt.

The substance, 4 gm., was dissolved in 2 liters of boiling alcohol, neutralized with 0.1 N alcoholic KOH, and a slight excess of an alcoholic solution of silver nitrate was added. An amorphous pure white precipitate separated immediately. The solution was cooled rapidly to room temperature and the precipitate was filtered and thoroughly washed with alcohol. After the precipitate was dried *in vacuo* over sulfuric acid, the snow-white powder weighed 4.2 gm. On exposure to light the substance darkened.

When heated in a capillary tube the substance sintered at 60° and liquefied at 160°, turning nearly black.

For analysis the substance was burned at a low heat in a porcelain crucible and the residue of metallic silver was weighed.

0.3769 gm. substance: 0.0294 gm. Ag.

Found. Ag 7.80.

The silver salt was insoluble in alcohol and in water. It was readily soluble in benzene and in chloroform and slightly soluble in ether. It was precipitated from its solutions by methyl alcohol.

Regeneration of Unsaponifiable Wax from Silver Salt.

The residual silver salt, 3.8 gm., was dissolved in 50 cc. of benzene by gentle warming. The perfectly clear solution was diluted with 75 cc. of ether and an excess of dry hydrochloric acid was added. The silver chloride was filtered off and washed with ether. The ethereal solution was washed with water until free from hydrochloric acid, dried with sodium sulfate, filtered, and concentrated by distillation to about 50 cc. The solution was mixed with 50 cc. of methyl alcohol and the substance was precipitated by cooling in ice water. It was filtered and reprecipitated from ether by methyl alcohol and cooling. The snow-white amorphous powder that was recovered weighed 3.5 gm. When heated in a capillary tube, it softened at 56° and melted at 57–58°. The substance was ash-free since 0.4248 gm. left no weighable residue on combustion. There was no loss in weight on drying at 105° *in vacuo* over dehydrite.

Analysis.

0.1062 gm substance: 0.1289 gm. H₂O and 0.3187 gm. CO₂.

Found. C 81.84, H 13.58.

The melting point and composition of the recovered substance were identical with that of the original material.

Acetyl Derivatives.

The substance, 2 gm., was refluxed with 300 cc. of acetic anhydride for 3 hours. The substance dissolved completely after the liquid had boiled for $\frac{1}{2}$ hour. When the solution was allowed to cool, a substance separated and formed a hard wax-like layer, which was filtered off and washed with alcohol. The acetic anhydride was evaporated to dryness under reduced pressure but practically no residue was left.

The acetylated product was twice precipitated from 50 cc. of ether by adding 50 cc. of cold methyl alcohol. The snow-white amorphous powder weighed 2.2 gm. after it had been dried *in vacuo* over sulfuric acid. The substance melted at 40° to a slightly opaque liquid which became transparent at 50°.

The acetic acid contained in the acetyl derivative was determined as follows: The substance was saponified with alcoholic potassium hydroxide, diluted with water, the alcohol removed by distillation, and the residue was acidified with phosphoric acid, and distilled until the distillate was practically neutral. The distillates were titrated with 0.1 N NaOH, with phenolphthalein as indicator.

Analysis.

0.7241 gm. substance: 9.25 cc. 0.1 N NaOH.

Found. CH_3COOH 7.66.

Another preparation gave the following value:

0.9850 gm. substance: 11.70 cc. 0.1 N NaOH.

Found. CH_3COOH 7.12.

When the substance was acetylated by the method of Einhorn and Hollandt (5) the following results were obtained. The acetyl derivative melted at 43–44° and after it had been saponified and distilled 0.5486 gm. of substance required 3.72 cc. of 0.1 N NaOH. Found, CH_3COOH 4.06.

It is evident, therefore, that the substance must contain either one or possibly two free hydroxyl groups. The acetyl derivative obtained by boiling with acetic anhydride contained nearly twice as much acetic acid as the one prepared by the use of acetyl chloride and pyridine.

Recovery of Unsaponifiable Wax from Acetyl Derivative.

The residues after the acetic acid was distilled off were extracted with ether, the ethereal solution was washed well with water, and the ether was distilled. The residue was twice precipitated from 50 cc. of ether by adding 50 cc. of cold acetone. The white amorphous powder melted at 57–58°.

Analysis.

0.1258 gm. substance: 0.1528 gm. H_2O and 0.3765 gm. CO_2 .

Found. C 81.62, H 13.59.

The melting point and composition are identical with those of the original material which would indicate that the substance was recovered unchanged.

The unsaponifiable wax is slightly volatile with steam. It was noticed during the distillations for the determination of the acetyl groups that a slight amount of water-insoluble material went over. When some of the original substance was boiled with water, it was noticed that a small amount of it volatilized with the steam.

This observation led to the experiments described below in which an attempt was made to distil the unsaponifiable wax under reduced pressure.

Distillation of the Unsaponifiable Wax.

The substance was placed in a Claissen flask provided with a wide outlet tube and the flask was heated in an air bath. The substance began to boil at 280° at a pressure of about 20 mm. The temperature was raised slowly and an oil distilled, mostly between 300 – 310° , which crystallized immediately in the outlet tube. The distillate was removed and it was recrystallized four times from benzene and once from acetone. It separated as thin colorless plates and melted when slowly heated at 87 – 88° , solidified at 86° , and remelted at 87 – 88° .

For analysis the substance was dried *in vacuo* at 61° over dehydrite but there was no loss in weight.

Titration.—0.2450 gm. of substance dissolved in neutral alcohol was titrated with 0.1 N alcoholic KOH, with phenolphthalein as indicator. Required, 6.14 cc. of 0.1 N KOH. Found, molecular weight 399.

Analysis.

0.0849, 0.0882 gm. substance: 0.1005, 0.1054 gm. H_2O and 0.2433, 0.2541 gm. CO_2 .

Found. C 78.89, 78.57, H 13.37, 13.37.

The analytical values would indicate that the substance is hexacosanic acid, $C_{26}H_{52}O_2$, but the melting point is several degrees higher than reported in the literature for cerotic acid. Calculated for $C_{26}H_{52}O_2$ (396), C 78.78, H 13.13.

Other samples of this acid, isolated after other distillation experiments, but which had not been so carefully recrystallized, showed melting points of 85° and 86°

Examination of Non-Volatile Residue.

The residue, after the high melting acid was distilled off, was a faintly yellowish soft sticky mass. It was dissolved in ether and, after a slight amount of insoluble matter was filtered off, the solution was precipitated by adding alcohol. These operations were repeated a second time. The substance was then completely soluble in ether. The clear ethereal solution was poured into 2 volumes of cold acetone, when a white amorphous precipitate separated. After the precipitate had been filtered, washed with acetone, and dried, it formed a somewhat sticky soft solid.

Titration.—0.4254 gm. of substance was not completely soluble in 50 cc. of boiling alcohol. Phenolphthalein and a drop or two of 0.1 N KOH were added, when a decided alkaline reaction was obtained. It is evident, therefore, that the substance has no acid properties.

For analysis the substance was dried at 61° *in vacuo* over dehydrite.

0.1648 gm. substance: 0.2020 gm. H₂O and 0.5055 gm. CO₂.

Found. C 83.65, H 13.71.

The fact that a high melting, saturated fatty acid could be isolated from the unsaponifiable wax by distillation under reduced pressure raised the question whether the acid was present as a contaminating substance or whether it was liberated under the influence of the high temperature. In an attempt to answer this question the following experiments were performed.

Fractionation of Unsaponifiable Wax by Precipitation.

The substance, 2 gm., was dissolved in 25 cc. of benzene by gentle warming, and on cooling to room temperature a gelatinous precipitate formed which was filtered and washed with cold benzene. These operations were repeated three times. After final drying of the precipitate, a white amorphous powder was obtained which weighed 0.9 gm. and which melted not sharply between 57–60°. The substance was again precipitated twice from warm benzene by cooling and twice from 50 cc. of ether by adding 50 cc. of cold methyl alcohol. The amorphous powder weighed 0.62 gm. and melted not sharply between 57–60°.

The material contained in the mother liquor was recovered by evaporating the solvents. The residue was twice precipitated from 50 cc. of ether by adding 50 cc. of methyl alcohol. The white amorphous powder melted at 55–56°.

While the seven precipitations, mentioned above, had caused a very slight change in melting point, yet both the top and the bottom fractions on titration with 0.1 N alcoholic KOH showed exactly the same neutralization value. It is evident, therefore, that no separation of an acid constituent had occurred.

An attempt to separate the unsaponifiable wax by fractional precipitation of the potassium salt was also ineffective. The product showed a slightly higher melting point, but the neutralization value was exactly the same as at first.

Action of Sodium Ethoxide upon Unsaponifiable Wax.

A sample of the unsaponifiable wax was refluxed for 24 hours with a solution containing 4 per cent of sodium in absolute ethyl alcohol. The principal portion of the substance was recovered after this treatment in an apparently unchanged condition. It melted at 56–57° and the neutralization value corresponded to a molecular weight of 1353. The results again indicated that practically no cleavage or saponification had occurred.

Action of Hydriodic Acid upon Unsaponifiable Wax.

The substance was heated in an ordinary Zeisel apparatus with hydriodic acid, sp. gr. 1.7, to 135–140° for 1 hour and 15 minutes and finally to 150–155° for 15 minutes. The volatile iodide was collected in an alcoholic solution of silver nitrate, converted into silver iodide in the usual manner, and weighed.

Analysis.

0.4563 gm. substance: 0.0464 gm. AgI. Found. Calculated as glycerol, 3.98.

In a second determination the substance was heated to 130–140° for 20 minutes and to 150° for 1 hour and 20 minutes.

Analysis.

0.5801 gm. substance: 0.0597 gm. AgI. Found. Calculated as glycerol, 4.03.

The results show that a volatile iodide is obtained when the unsaponifiable wax is boiled with hydriodic acid. If the substance that is formed is isopropyl iodide, the amount obtained corresponds to about 4 per cent of glycerol, but up to the present time we have not been able to determine the nature of the iodide.

The residues from the Zeisel determinations formed a wax-like mass which was filtered off, washed with water, and dried *in vacuo* over sulfuric acid and potassium hydroxide. The substance, which weighed 1.1 gm., was precipitated four times from ether by adding methyl alcohol. The purified material was found to contain iodine. It was a white amorphous powder which weighed 1 gm. and it melted at 46–47°.

Analysis.

0.1459 gm. substance: 0.1516 gm. H₂O and 0.3805 gm. CO₂.

0.2858 " " : 0.0553 " AgI.

Found. C 71.12, H 11.62, I 10.45.

The values found on analyzing the unsaponifiable wax do not lead to any well defined simple formula. The average of five closely agreeing analyses gave C 81.70 and H 13.60 per cent. The relation of carbon to oxygen is, therefore, about 23.2:1. However, if the substance contains one carboxyl and two hydroxyl groups, then the simplest formula would be represented by C₉₃H₁₈₆O₄ or C₉₄H₁₈₈O₄. The values previously given for titration with 0.1 N KOH, the composition of the potassium salt, the silver salt, and the acetyl derivatives agree approximately with the formulas mentioned above.

It was unfortunately impossible to make any direct determinations of the molecular weight of the substance. In the boiling point method with benzene or ether as solvents a depression of the boiling point was observed rather than a rise. In the freezing point method with naphthalene as solvent values from 1711 to 1824 were obtained, while the method of Rast gave a value of 1046 for the molecular weight.

As mentioned in a former paper (1) the unsaponifiable wax is acid-fast¹ and it is the only fraction of the lipoids of the tubercle

¹ These determinations were made by Professor W. L. Kulp, Department of Bacteriology, Yale University, and by Dr. M. C. Kahn, Cornell University Medical College.

bacilli that we have isolated which shows the property of acid fastness.

SUMMARY.

The so called unsaponifiable wax obtained from tubercle bacilli possesses both acid and alcoholic properties. It decomposes on heating, yielding a higher fatty acid and a neutral non-volatile substance.

When heated with hydriodic acid, the substance decomposes and a volatile iodide is liberated which calculated as isopropyl iodide would be equal to 4 per cent of glycerol.

The substance is optically and biologically inactive.

The unsaponifiable wax is the only fraction isolated from the fat of tubercle bacilli which is acid-fast.

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THE CHEMISTRY OF THE LIPOIDS OF TUBERCLE BACILLI.

IX. THE OCCURRENCE OF HEXACOSANIC ACID IN THE UNSAPONIFIABLE WAX.*

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INTRODUCTION.

In a study of the unsaponifiable wax (1) obtained from the human type of tubercle bacillus, Strain H-37, it was found, on heating the substance under reduced pressure, that a saturated fatty acid distilled at about 300°. The purest specimen of this acid that we obtained melted at 87–88° and its molecular weight and composition indicated that it was hexacosanic acid but at the time of the former investigation the acid had not been identified.

In the meantime an all-glass distilling apparatus (2) had become available in which substances could be distilled in a very high vacuum. Some of the unsaponifiable wax was therefore distilled in this apparatus in the hope that better yields as well as a purer product might be obtained. The results were, however, essentially similar to those found in the first experiments with the cruder apparatus.

The distillate was purified by crystallization from benzene, acetone, and a mixture of ether and methyl alcohol. The acid crystallized usually in thin colorless plates but from ether and methyl alcohol it separated in needles. The purified substance melted at 88–89°, solidified at 85°, and remelted at 88–89°. The molecular weight and the composition of the free acid agreed with the calculated values for hexacosanic acid, $C_{26}H_{52}O_2$.

* The present report is a part of a cooperative investigation on tuberculosis and it has been supported partly by funds provided by the Research Committee of the National Tuberculosis Association.

As a final step in the identification of the acid its melting point was compared, through the kindness of Dr. P. A. Levene of the Rockefeller Institute, with the melting point of the synthetic hexacosanic acid prepared by Levene and Taylor (3). Dr. Levene described these melting points as follows: "Our substance melts at 88.5–90° and solidifies at about 85.5–85°. Yours melts at 87–87.5°, solidifies at 83.5°. Mixed melting point 88–89.5°; solidifies at 85.5–84°. I think your substance is slightly impure, otherwise identical with the synthetic C_{26} ."

It appears then to be reasonably well established that an acid, identical with the synthetic hexacosanic acid, is formed when the unsaponifiable wax is heated either in very high vacuum or at a pressure of about 20 mm. We have, unfortunately, no additional information regarding the neutral non-volatile substance which remains after the acid has been distilled.

EXPERIMENTAL.

The unsaponifiable wax, 10 gm., was introduced into a 100 cc. round bottom Pyrex flask and the flask was fused onto the fractionating column of the distillation apparatus. The system when evacuated showed a pressure of about 0.0001 mm. as measured on the MacLeod gage. The flask was heated in an air bath, a temperature of 280–290° being maintained. The distillate began to come over at 231° but the temperature rose rather quickly to 250°. Towards the end, the temperature of the bath was increased to 345–350°, when the last small fraction of the distillate went over at 275°. During the distillation the condenser was heated by passing an electric current through the nichrome band in order to keep the distillate from solidifying in the outlet tube.

The distillate, which was faintly yellow in color and weighed 3.2 gm., was dissolved in 40 cc. of hot benzene. Colorless plate-shaped crystals separated as the solution cooled. The substance was recrystallized three times from benzene, and twice from acetone. The colorless crystals weighed 1.1 gm. and when slowly heated, melted at 88–89°, solidified at 85°, and remelted at 88–89°.

For further recrystallization the acid was dissolved in 100 cc. of warm ether and the solution was diluted with 50 cc. of methyl alcohol. When the solution was cooled, the acid crystallized in small colorless needles. The crystals were filtered off, washed

with methyl alcohol, and dried *in vacuo* over sulfuric acid. The substance was snow-white and weighed 0.9 gm. The melting point was the same as before.

For analysis the acid was dried at 61° *in vacuo* over dehydrite but there was no loss in weight.

0.1131 gm. substance: 0.1353 gm. H_2O and 0.3271 gm. CO_2 .
 $C_{26}H_{52}O_2$ (396). Calculated. C 78.78, H 13.13.
Found. " 78.87, " 13.38.

Potassium Salt.

The acid, 0.4198 gm., was dissolved in 100 cc. of hot absolute alcohol, phenolphthalein was added, and the solution was titrated with 0.1 N alcoholic KOH. Required, 10.46 cc. of 0.1 N KOH. Molecular weight, 399. The calculated molecular weight for $C_{26}H_{52}O_2$ is 396.

The neutralized alcoholic solution on standing at room temperature deposited the potassium salt as a white precipitate. The substance was filtered off, washed with alcohol, and dried *in vacuo* over sulfuric acid. It weighed 0.4 gm. Heated in a capillary tube it fused and darkened without completely melting at 230° . For analysis it was burned at a low heat in a platinum crucible.

0.1394 gm. substance: 0.0220 gm. K_2CO_3 .
 $C_{26}H_{51}O_2K$ (434.1). Calculated. K 9.00.
Found. " 8.93.

Silver Salt.

The potassium salt, 0.2 gm., was dissolved in 50 cc. of hot alcohol and a slight excess of an alcoholic solution of silver nitrate was added. The silver salt separated as a white, somewhat gelatinous precipitate. It was filtered off, washed thoroughly in alcohol, and dried *in vacuo* over sulfuric acid. The weight of the dry substance was about 0.2 gm. When the salt was heated in a capillary tube, it sintered between 130 – 140° , turned dark between 190 – 200° , and melted to a brown liquid at 210° . The substance was insoluble in alcohol, water, or benzene. It did not lose in weight on drying at 61° *in vacuo*.

Analysis.

0.1414 gm. substance: 0.0306 gm. Ag.

$C_{26}H_{51}O_2Ag$ (502.88).	Calculated.	Ag 21.45.
	Found.	" 21.64.

In conclusion we desire to acknowledge our indebtedness to Dr. Gösta Åkerlöf for assistance in the operation of the distillation apparatus and to Dr. P. A. Levene for comparing the melting point of our product with the synthetic hexacosanic acid.

SUMMARY.

The unsaponifiable wax obtained from tubercle bacilli when distilled in a high vacuum decomposes with the liberation of hexacosanic acid.

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INORGANIC ELEMENTS OF SPINACH IN THE TREATMENT OF NUTRITIONAL ANEMIA.*

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Within the last 18 months there have developed two theories concerning the inorganic factors which influence hematopoietic processes. (1) In rats on a basal diet of milk and iron, supplementary copper alone will stimulate a maximum hemoglobin production. (2) On such a diet a group of elements including copper produces a maximum hemoglobin response while copper alone is slightly less effective. By comparing the effect of liver extract, H_2S fraction of the extract, and copper sulfate solutions on hemoglobin of rats, Hart and coworkers (1) have concluded that in the milk-iron diet inorganic copper is the only deficiency. However, Titus and coworkers (2, 3) have found that the rapidity of hemoglobin regeneration is greatly increased by the addition of copper-free manganese chloride to the milk-iron-copper diet and that there is a storage in the body of both copper and manganese which is later effective in the utilization of iron. Recently Myers and Beard (4) have reported that nickel, cobalt, and germanium as well as copper are effective in shortening the hemoglobin regeneration period.

In attempting to determine whether elements other than iron and copper influence hematopoietic function, it occurred to us that a clue to the proper balance of inorganic constituents might be found in the combination of minerals naturally present in a food generally conceded to be a good blood builder. If a material of natural source which supplies 0.5 mg. of iron and small quantities of other salts would bring about the regeneration of hemo-

* Read before the Division of Biological Chemistry at the Seventy-Eighth Meeting of the American Chemical Society, Minneapolis, September 9-13, 1929.

globin in a shorter time than would equivalent quantities of iron and of copper in the form of pure salts, it would appear that other factors must be involved in this metabolic process.

For this purpose a concentrated water extract of dried spinach¹ was used and the results of this work are presented in this paper.

EXPERIMENTAL.

In order to make the proper dilutions for feeding the extract in quantities which would supply 0.5 mg. of iron, the iron content was determined. At the same time quantitative determinations of the copper and manganese were made, it being realized that at least one and possibly both were significant. The ratio of the iron, copper, and manganese was found to be 39:1.1:1, respectively.

Young rats in which a condition of anemia had been induced by limiting the mother's diet to milk and wheat germ for several months, as has been described in previous publications from this laboratory (5, 6), were used when about 4 weeks of age. At that time, supplements were added to the whole milk diet unless the hemoglobin content of the blood was not sufficiently low. In that case, the mineral supplement was withheld for several weeks or until the hemoglobin readings were 6 gm. per 100 cc. of blood or below. Negative controls from each litter were maintained on milk alone or milk and pure iron salts.

Weekly weight and hemoglobin records were kept. Growth was always retarded on the milk ration but approached the normal in all animals receiving adequate mineral supplement. Variations in the weight curves were quite irrelevant to the type or amount of minerals added. For hemoglobin determinations, the regular acid hematin method with a Bausch and Lomb hemoglobinometer was followed. However, to insure maximum color development, the acid hematin samples were heated for 7 minutes by being placed in a water bath at 55–60° and then were cooled to room temperature.

The results of some earlier work with spinach extract had shown that animals receiving this extract in doses supplying 0.8 mg. of iron recovered very rapidly. In order to ascertain whether the rapid recovery had been due to the excessive quantity of iron administered or to presence of other elements, investigation of

¹ Prepared by the Battle Creek Food Company.

smaller dosages was made. A litter of rats was divided into two groups. As daily supplement (6 days per week) those rats in Group I were fed a quantity of spinach extract that furnished 0.5 mg. of iron and those in Group II were given but half that dosage. Since 0.25 mg. of iron is below the quantity that has been considered an optimum in most studies, it was expected that the recovery of the animals in Group II would be slower than for those of Group I. But as may be seen in composite Table I, it required but 1 week longer for the hemoglobin of the animals in

TABLE I.

Average Hemoglobin Levels at Time Supplement Was Added and at Intervals Thereafter, of Animals Receiving 0.5 and 0.25 Mg. of Fe from Spinach Extract, Ash, and HCl Solution of Ash.

Figures represent gm. of hemoglobin per 100 cc. of blood.

	0.5 mg. Fe from spinach extract.	0.25 mg. Fe from spinach extract.	0.5 mg. Fe from ash of extract.	0.25 mg. Fe from ash of extract.	0.5 mg. Fe from HCl solution of ash.
At time of addition.	5.9 (7)*	6.0 (4)	4.2 (4)	4.2 (5)	4.4 (5)
After addition.					
1 wk.	12.1 (6)	9.2 (4)	5.8 (4)	4.9 (5)	10.9 (5)
2 wks.	15.4 (6)	11.7 (4)	7.5 (4)	5.1 (5)	13.0 (5)
3 "	15.1 (7)	14.0 (4)	7.6 (4)	5.7 (5)	13.9 (5)
4 "	16.9 (7)	14.6 (5)	9.7 (4)	5.7 (5)	16.5 (5)
5 "	16.1 (7)	15.2 (5)	11.5 (4)	6.9 (5)	15.7 (5)
6 "	16.3 (6)	16.6 (5)	14.6 (4)	7.2 (5)	15.1 (5)
7 "	17.1 (6)	16.4 (5)	16.0 (4)	7.6 (5)	15.4 (5)
8 "	17.0 (6)	16.4 (5)	14.0 (4)	9.1 (5)	16.6 (5)

* The figures in parentheses indicate the number of animals used in arriving at the average figure.

Group II to regenerate, as compared to the animals of Group I, the average being 2.1 and 3 weeks, respectively.

As a means of comparison, 14 gm. of hemoglobin per 100 cc. have been chosen as an indication of a fair degree of regeneration although not as high as for our normal rats. The hemoglobin levels reached in most of the animals were well above this figure. For simplicity, we shall speak of it as having been regenerated when the hemoglobin has increased to 14 gm. or more per 100 cc. of blood.

The question arose of whether an organic factor might be

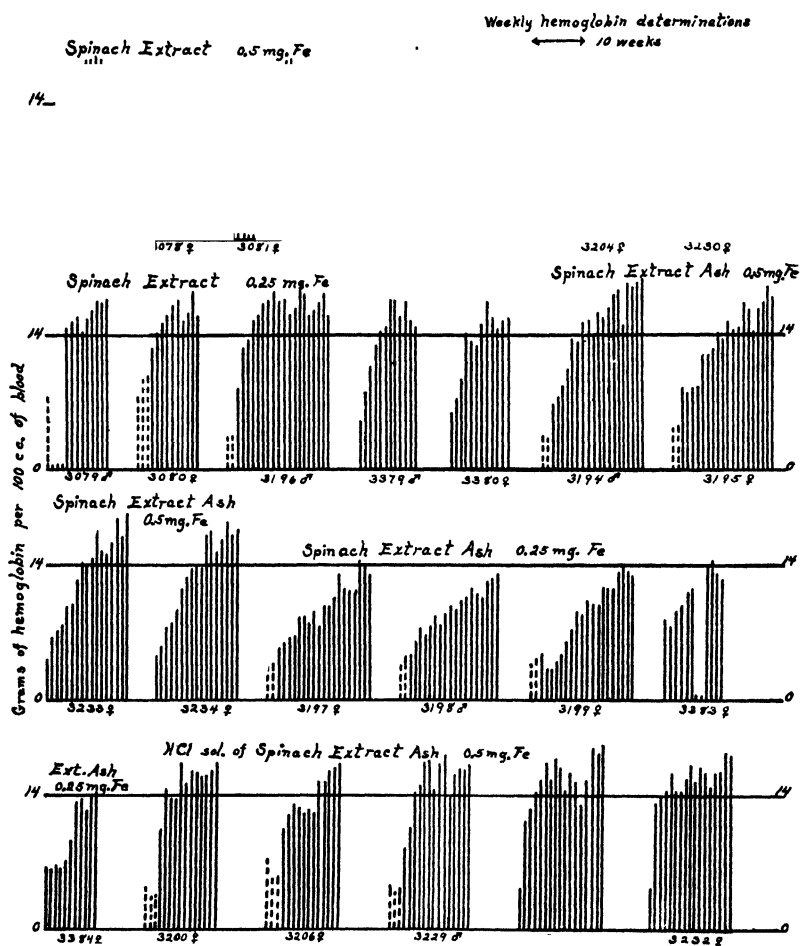


CHART I. Hemoglobin response of rats receiving a milk ration¹ plus mineral supplement in the form of spinach extract or its ash. The broken line is used for the period on milk alone. 14 gm. of hemoglobin per 100 cc. of blood were chosen as indicative of a fair degree of blood regeneration although below the optimum.

involved; hence, the first step was the destruction of the organic matter by means of ashing the spinach extract and the subsequent feeding of this ash in graded quantities (0.5 mg. and 0.25 mg.). Since this ash was insoluble in water or milk, it was necessary that great care be exercised in feeding it. Complete consumption was secured by repeatedly mixing the ash with small amounts of milk until the dish had been licked clean before feeding larger quantities of milk.

The rats receiving 0.5 mg. of iron in the form of ash were much slower in responding than those receiving an equivalent quantity

TABLE II.

Average Hemoglobin Levels at Time Supplement Was Added and at Intervals Thereafter, of Animals Receiving a Basal Diet of Milk and Iron with 0.05 Mg. of Copper, or 0.05 Mg. Each of Copper and Manganese.

Figures represent gm. of hemoglobin per 100 cc. of blood.

	0.05 mg. Cu as sulfate.	0.05 mg. Cu and 0.05 mg. Mn as sulfates.
At time of addition.	4.2 (25)*	6.3 (9)
After addition:		
1 wk.	6.0 (24)	7.3 (9).
2 wks.	8.0 (25)	9.8 (9)
3 "	9.8 (24)	12.5 (8)
4 "	11.3 (23)	14.6 (9)
5 "	12.4 (25)	14.8 (7)
6 "	13.4 (20)	15.5 (9)
7 "	13.8 (24)	15.4 (9)
8 "	13.8 (25)	15.1 (9)

* The figures in parentheses indicate the number of animals used in arriving at the average figure.

of the spinach extract, and in fact slower than those receiving but one-half dose of the extract, 6 and 9 weeks being required for regeneration, as compared with 2 and 3 weeks previously mentioned. It was also noted that the hemoglobin content of the animals receiving but 0.25 mg. of iron as the ash either did not reach or exceed the level of 14 gm. during the whole experimental period of 19 weeks, whereas on an equivalent quantity of the extract the hemoglobin attained levels of 18 or 19 gm. per 100 cc. of blood.

The much slower response of those rats receiving the ash supple-

ment might be taken as indicative of an organic factor involved in the spinach extract. Previous experiences, however, had suggested that the insoluble nature of the ash might alone be responsible for the longer regeneration period. Therefore, a solution was prepared by digesting the ash with dilute hydrochloric acid until completely dissolved which after further dilution could be fed in quantities supplying 0.5 mg. of iron.

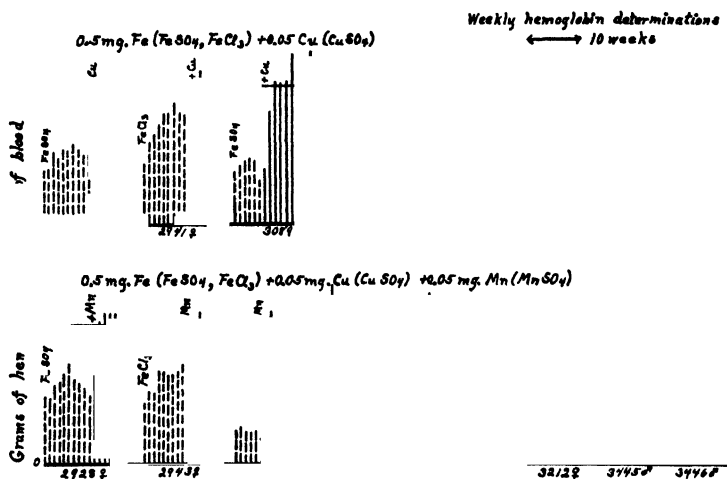


CHART II. Hemoglobin response of rats receiving a milk ration plus mineral supplements in the form of carefully purified salts. The broken line is used for the period when iron alone supplemented the milk ration; the solid line, when Cu or Cu and Mn were added. 14 gm. of hemoglobin per 100 cc. of blood were chosen as indicative of a fair degree of regeneration although below the optimum.

The difference in the potency of the ash and the solution of the ash was very marked, the time for regeneration being 2 to 3 weeks longer for the ash than for the solution, whereas there was much less difference in the potency of the original spinach extract and the solution of the ash (Chart I). Thus from these preliminary data accumulated on twenty-six animals, we feel warranted in concluding that the potency of the hemoglobin-building material in spinach extract is increased by being in solution and that the active principle in the spinach extract must be of inorganic nature.

The quantities of copper and manganese carried by the extract

were 0.014 and 0.012 mg., respectively, corresponding to 0.5 mg. of Fe and 0.007 and 0.006 mg. corresponding to 0.25 mg. of Fe. In all the work on the supplementary value of copper and manganese salts, 0.05 mg. was the standard dose and the average hemoglobin levels of rats receiving these salts are shown in Table II. In a group of twenty-five animals receiving iron and copper the shortest regeneration period was 3 weeks, the longest 12 or more, and the average about 8 weeks. This recovery period averaged 4 times longer than that of the animals receiving iron from the spinach extract and $2\frac{1}{2}$ times longer than those receiving the HCl solution of the spinach extract ash. When copper and manganese together were fed with the iron to nine rats, the recovery period was somewhat shorter than with copper only (Chart II). The HCl solution of spinach extract ash, however, is still better than any combination of pure salts yet tried.

A qualitative analysis² of the extract has been made and the following elements were found to be present: copper, antimony, tin, iron, aluminum, zinc, manganese, strontium, sodium, potassium, calcium, magnesium, and phosphorus. The quantitative relations of these elements and their significance in hemoglobin building is being studied further. In order to determine whether these metals are normally present in spinach or whether the vessels and cans used in the course of preparing and storing the product are the source of some of the elements, the ash of local New Zealand spinach has been subjected to a similar analysis. Antimony, tin, strontium, and zinc were not present in this spinach.

DISCUSSION.

The rapid increase in the hemoglobin content of the blood of animals receiving but 0.25 mg. of iron from the extract indicates that the daily iron requirement may be less than 0.5 mg. in the presence of other metals such as copper, manganese, etc. Of course, in the early work on nutritional anemia, when the customary iron dosage was established as 0.5 mg. the rôle of copper and other elements was unknown. Small traces of these metals in the iron salts or the storage of these elements in the body may account for the results obtained by using that quantity of iron.

² The assistance of Russell B. Cooper in checking these analytical data is hereby acknowledged.

There is a marked difference in the potency of equal quantities of the ash depending upon whether it is administered in the form of a solid or in the form of a solution, the latter showing distinctly better utilization. The very slow response of the animals on even half dosage of ash must, however, be attributed to a partial utilization of insoluble material rather than to any spontaneous recovery since such did not occur in the control group. It is theoretically probable that a portion of this ash may become dissolved during the process of digestion and thus become available.

When 0.05 mg. of copper was used as a supplement to the milk-iron diet, the recovery of the animals was slower than for either dosage of spinach extract or for the HCl solution of the ash. This quantity of copper is about 4 and 8 times as great respectively as that supplied by the two dosages of extract in its various forms. According to the data recently reported by Waddell, Steenbock, Elvehjem, and Hart (1) there is a shortening of the regeneration period with an increased copper dosage. If copper is the only deficiency in the basal milk-iron diet it is difficult to explain why the smaller dosages of copper in the extract should be more effective than the larger doses of copper as the sulfate.

Furthermore, the increased response on a combination of copper and manganese over and above that of copper alone is another point which may add weight to the theory that there is a group of elements that play a rôle in the regulation of hemoglobin building.

Robscheit-Robbins and coworkers (7) have reported that they have been unable to duplicate with artificial salt mixture the balance of inorganic salts in liver, kidney, and apricot ashes, which are effective in hemoglobin building. Furthermore, McHargue (8) has shown that iron, copper, manganese, and zinc as well as phosphorus, calcium, potassium, and sodium are contained in both cow's blood and calf's liver. All of these elements and additional ones are present in the spinach extract studied. It seems possible that at least some of the other elements present may have an influence as well as the iron and copper and that there may be an optimum balance of inorganic salts for hemoglobin regeneration. Further studies of this phase of the problem are now under way.

SUMMARY.

Spinach extract when used as a supplement to a milk diet for anemic animals in such quantities as to supply 0.5 mg. of iron, 0.014 mg. of copper, and 0.012 mg. of manganese or in half that quantity proved to be more potent than an iron-copper or iron-copper-manganese complex of pure salts in the building of hemoglobin.

Salts in solution are more effective than in the insoluble form as equal quantities of the spinach extract ash in these two states showed very marked differences in hematopoietic powers.

The analysis of the extract shows that Cu, Sb, Sn, Fe, Al, Zn, Mn, Sr, Ca, Mg, Na, K, and P are present.

Experimental data indicate that the daily iron requirement for a rat is less than 0.5 mg. if properly supplemented and that rather than copper alone there is a group of elements that is active in hemoglobin building.

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THE INTERCONVERSION OF HEXOSES BY MEANS OF PHOSPHATES AND THE FORMATION OF GLUTOSE.

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Lobry de Bruyn and Van Ekenstein (1) discovered in 1895 that when either *d*-glucose, *d*-mannose, or *d*-fructose is treated with a solution of an alkali, a mixture of all these sugars results together with *d*-glucose. Similar results were obtained with *d*-galactose which yielded a mixture of this sugar and *d*-talose, *d*-tagatose, *l*-sorbose, as well as *d*-galtose. This effect of alkalies on the hexose sugars has been the subject of extensive investigations, especially by Nef, more recently by Lewis and his collaborators and by others¹ (2-6). The immediate objectives of the more recent investigations of the Lobry de Bruyn-Van Ekenstein reaction have been two (7-12). One class of investigations has been designed to discover the chemical mechanism underlying the reaction, being thus more directly of theoretical chemical significance. In another class of investigations the reaction has been studied with a view to its significance in the interpretation of carbohydrate transformations in the living organism, both plant and animal. While obviously these two classes of investigation are interrelated, the view-points and experimental conditions may vary considerably. This is true, for example, of the hydroxyl ion concentration employed in the different experiments. The biologist is more directly concerned with the reactions and conditions which most nearly simulate those occurring in the living organism. The theoretical chemist has often been quite unmindful of this aspect of the problem. Much of the theoretical significance which attaches to the Lobry de Bruyn-Van Ekenstein reaction from the biological

¹ Because of the indefinite nature of glucose and galtose, the literature on the subject is rather confusing. Nef ((2) 403, 239, 360, 374) regarded α - and β ,*d*-galtose and α - and β ,*d*-glucose as 3-ketohexoses and as being quite different from the compounds to which Lobry de Bruyn and Van Ekenstein gave the names galtose and glucose. Nef considered that Lobry de Bruyn and Van Ekenstein's compounds were *o*,*d*-galactosone and *o*,*d*-glucosone. It should be pointed out, however, that Nef never actually isolated in crystalline form nor established the structure of his 3-ketohexoses, nor did he prove that the galtose and glucose of Lobry de Bruyn and Van Ekenstein were osones.

view-point may be traced to Baeyer's theory of sugar dissociation in vital processes, such as fermentation (13, 14). He postulated the splitting out of water from one place in the hexose molecule and the subsequent addition of water in another place. Moreover, the Lobry de Bruyn-Van Ekenstein reaction constitutes the only chemical point of contact with the very important, though still entirely unexplained, interconversions of the carbohydrates occurring in plants (15-27).

This investigation was prompted primarily by the desire to determine what is the first sugar formed in photosynthesis. But the solution of this problem must await an elucidation of the manner in which the simpler carbohydrates undergo interconversion *in vitro* and in the plant independent of any photosynthesis of carbohydrates. Also, because of the fact that alkalis cause splitting of the hexose molecule it has been the hope that a knowledge of these reactions may throw some light on the nature of the intermediate products of sugar metabolism and on the mechanism of this phenomenon in plants (18).

In most of the earlier investigations referred to relatively strong alkalis were used; *viz.*, potassium hydroxide, calcium hydroxide, barium hydroxide, lead hydroxide, etc. Besides the interconversion of the sugars, these reagents cause more drastic changes in the sugar molecule. In order to avoid these secondary changes Nef² employed sodium carbonate and he considered that with this reagent the change in the sugar molecule was confined to the interconversion action. Henderson (19) observed that glucose in solutions of mixtures of disodium phosphate and monosodium phosphate at 38° slowly decreased in optical rotation. Dakin and Dudley (20) as well as Neuberg and Oertel (21) found that methylglyoxal is formed on heating solutions of glucose with disodium phosphate. Recently Spöchr and Wilbur (22) have shown that *d*-glucose and *d*-fructose undergo the Lobry de Bruyn-Van Ekenstein reaction with solutions of disodium phosphate at 38° and that under these conditions *d*-glutose is also formed.

- The action of alkalis on the monosaccharides when carried out in the absence of oxidizing agents is characterized by the following: (1) hastening of the mutarotation phenomenon, (2) decrease in the optical activity of the sugar solution which finally attains a value very close to zero, (3) decrease in the reducing power of the solu-

² Cf. (2) 403, 239, 340, 353, 369.

tion toward cuproalkaline solutions, (4) tar formation as evidenced by yellow to brown coloration, and (5) in the case of high hydroxyl ion concentration, the formation of acids of the general formula $C_nH_{2n}O_n$, the saccharinic acids. Included in the latter is the well known formation of lactic acid. The action of alkalis on hexose sugars is very materially influenced by the hydroxyl ion concentration and by temperature. An examination of the resulting products indicates that there are distinct types of reactions occurring: (1) an interconversion of the isomeric hexoses, (2) the formation of saccharinic acids, and (3) the splitting of the hexose molecule with subsequent rearrangements.

Interconversion in the Glucose Group: Composition of "Equilibrated Mixtures."

The investigations hereinafter described are concerned primarily with the effect on *d*-glucose, *d*-mannose, and *d*-fructose of relatively weak alkalis, sodium carbonate, disodium phosphate, and mixtures of the latter with potassium dihydrogen phosphate. Under these conditions secondary reactions, *e.g.* saccharinic acid formation, are at a minimum. The final mixture of sugars resulting from the action of an alkaline solution on any one hexose has been spoken of as an "equilibrated mixture," implying that a state of equilibrium between the different sugars is attained by starting with any one of the hexoses concerned. On the basis of the Wohl-Nef theory the interconversion of the monosaccharides is interpreted by the formation of intermediate enediols, one of which for example is common to *d*-glucose, *d*-mannose, and *d*-fructose and through rearrangement can yield any of these isomeric compounds. A valuable contribution to this theory has been made by Lewis and his collaborators from a study of the interconversion of methylated sugars.

As Nef³ pointed out, it is impossible to obtain a true equilibrium with hexoses and the relatively strong alkalis due to the fact that saccharinic acid formation from hexoses is an irreversible reaction. Although the secondary reactions involving the formation of saccharinic acids are largely obviated by the use of relatively low hydroxyl ion concentration, *e.g.* disodium phosphate, the rate of the interconversion is lower as compared to that when

³ Cf. (2) 357, 296.

stronger alkalis are used. This together with the fact that the reactions were carried out at relatively low temperatures, 25° and 37° in most of the experiments, accounts for the extended periods of time required to attain conditions approaching equilibrated mixtures. It would be of considerable interest to determine exactly how the mixtures of sugars ultimately formed when either *d*-glucose, *d*-mannose, or *d*-fructose is used to start with differ in composition.

General Procedure and Analytical Methods.

In the following experiments 100 gm. of the respective hexose sugars were dissolved in 1.7 times their weight of water and in another flask 83.3 gm. of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ were dissolved in an equal weight of water. These solutions were sterilized in an autoclave at 15 pounds for 20 minutes, and, after cooling, the sugar solution was added to the phosphate solution, the flask being rinsed with sterilized water. The total amount of water was 333.3 gm. As an antiseptic agent 2.5 gm. of xylene were added to each flask. This was tightly stoppered and placed in a thermostat kept at 37°. The first sample for analysis (zero time) was taken after 1 hour by withdrawing 15 cc. of the solution with a sterilized pipette. This was weighed and then diluted to a suitable volume for analysis.

The analytical methods were the same as those employed in the earlier experiments of Spoehr and Wilbur already referred to. Determinations were made of the following: the total reducing power of the mixture by means of the Benedict reagent, the aldoses by means of the method described by Cajori (23) with an oxidation period of 1 hour, mannose by precipitation as the phenylhydrazone ((4) p. 843, (24)) in dilute acetic acid solution below 5°. A portion of the solution was first treated with a saturated solution of lead acetate, enough to precipitate the phosphate, and the lead phosphate separated by centrifuging. The mannose was determined in the clear supernatant solution. The insoluble *d*-mannose phenylhydrazone was collected on tared Jena glass filters, dried, weighed, and the purity tested by the melting point, and in some cases by the determination of the nitrogen content by means of combustion. The optical rotation was determined in a 2 dm. tube at 16°. The reserve alkali was determined by titration with

0.1 N hydrochloric acid with methyl orange as indicator. The hydrogen ion concentration was determined electrometrically in duplicate, by use of Clark cells with saturated KCl-HgCl-Hg as the reference electrode. The electrodes were checked against 0.05 molal potassium acid phthalate.

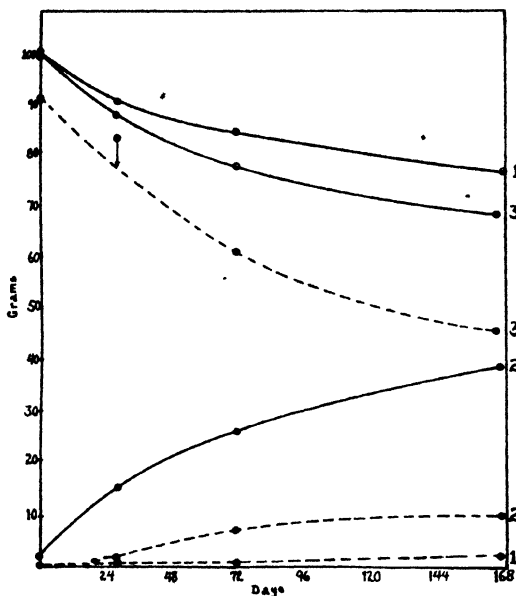


FIG. 1. Effect of disodium phosphate on solutions of hexose sugars at 37°. The solid lines indicate change in aldose content, the broken lines change in mannose content of solutions of *d*-glucose (Curves 1), of *d*-fructose (Curves 2), and of *d*-mannose (Curves 3).

Experimental Results.

From the experimental results shown in Fig. 1 and Table I it can be seen that although the hydroxyl ion concentration of these solutions was low there was a very appreciable change in their composition. Owing to the fact that the reducing power of some of the products formed is not known with certainty, *e.g.* glucose, it is not possible to calculate very accurately the true composition of the mixture. However, at the conclusion of the experiment approximately 20 per cent of the *d*-glucose originally present had

been converted into ketoses; *i.e.*, about 80 per cent were aldoses. In the *d*-mannose experiment approximately 22 per cent was converted to ketoses. In the case of *d*-fructose about 40 per cent had been converted into aldoses. This sugar undergoes interconversion far more rapidly than either of the aldoses; *d*-mannose also undergoes interconversion more rapidly than *d*-glucose. From the calculated results given in Table II it can be seen that the composition of the three sugar mixtures differed considerably even after 167 days at 37°.

TABLE I.

Change in Total Reducing Power, Optical Rotation, Free Base, and Hydrogen Ion Concentration in Solutions of Hexoses Treated with Disodium Phosphate at 37°.

	Time.	Total reducing power.	$[\alpha]_D^{16}$	Free base as HCl.	pH
	<i>days</i>		<i>degrees</i>	<i>gm.</i>	
<i>d</i> -Glucose.	0	99.2	+48.3	8.59	8.28
	27	99.9	+34.6	8.43	7.88
	71	100.0	+24.4	8.38	7.64
	168	97.3	+19.4	8.23	7.42
<i>d</i> -Fructose.	0	102.7	-86.8	8.68	8.20
	27	99.0	-59.2	8.41	7.59
	71	97.4	-43.3	8.32	7.45
	167	94.2	-26.6	8.19	7.10
<i>d</i> -Mannose.	0	85.0	+12.1	8.80	8.16
	27	89.7	+2.7	8.54	7.74
	71	90.2	-6.68	8.45	7.57
	166	89.8	-10.46	8.20	7.35

A significant question in all experiments which have dealt with the Lobry de Bruyn-Van Ekenstein reaction is whether under the experimental conditions employed the reaction is ever confined to an interconversion of hexose sugars or whether also other reactions do not always occur. Moreover, if such secondary reactions occur, what would be their influence on the hexose interconversion? Although Groot,⁴ from his experiments with dilute solutions of potassium hydroxide concluded that in the tendency of alkaline

⁴ *Cf.* (8) 146, 80.

hexose solutions to become optically inactive there is involved only the rearrangement of hexoses, it must be borne in mind that such solutions decrease decidedly in hydroxyl ion concentration. Recently this has again been shown by Wolfrom and Lewis ((4) p. 842), who, in determining the quantitative interconversion of *d*-glucose into other hexoses with calcium hydroxide, found that the solution became neutral. From the evidence available, especially from the work of Nef, it seems most probable that this neutralization of the free alkali is due to the formation of saccharinic acids. When the concentration of the alkali is relatively high, all of the sugar is converted into acids containing 1 to 6 carbon atoms.⁵ With lower concentrations of alkali the formation of acids is less and the remaining sugar mixture gives an indication of the interconversion action which has gone on simultaneously.

TABLE II.

Approximate Composition of Final Mixtures on Assumption That Only Aldoses Present Are Glucose and Mannose.

	Aldoses.	Ketoses	Mannose.	Glucose.
	per cent	per cent	per cent	per cent
<i>d</i> -Glucose.	78	22	2	76
<i>d</i> -Mannose.	68	32	46	22
<i>d</i> -Fructose.	39	71	10	29

It has commonly been assumed that the interconversion is due to an enolization of the sugar molecule in alkaline solution or to the formation of an unstable salt, *e.g.* sodium glucosate, the velocity of the reaction being dependent directly on the concentration of the free hydroxyl ions in the solutions. From an examination of the rates of the interconversion action in dilute alkaline solution it becomes evident that these decrease with time. From the experimental evidence available it is impossible to establish whether this decrease is due to the fact that the mixture is approaching a condition of equilibrium of hexose components or whether it is due to the fact that because of acid formation the

⁵ That the enediols, presumably formed as intermediate products, have acid properties and form stable salts suggests itself as an alternate theory, but lacks substantiating evidence.

hydroxyl ion concentration decreases correspondingly.⁶ Thus the final composition of a sugar mixture would depend upon a number of factors. These would include the concentration of the hydroxyl ions in the solution, the relative rate of acid formation as compared to interconversion, and the relative rates of acid formation from the different sugars present. As a matter of fact, various workers have found widely different proportions of the sugars in the final mixtures. Another fact must also be taken into consideration in this connection. That is, that, contrary to the opinion of Nef, glucose, mannose, and fructose, or their enediols, undergo splitting even in weakly alkaline (or neutral phosphate) solution (20, 21, 25). This probably results in the formation of glyceraldehyde, dihydroxyacetone, and methylglyoxal, which in turn condense to optically inactive hexose sugars which may be more stable than the naturally occurring hexoses (26, 27). The fundamental causes underlying the differences in reactivity of the various isomeric hexoses is of great significance to biology. Whether this is associated with the degree of asymmetry of the molecule or with what property thereof, no physical-chemical methods have as yet revealed.

From the results of our experiments with the weakly alkaline disodium phosphate, Table I, it is evident that these solutions had not reached a condition which has been described as an equilibrated mixture even after 168 days at 37°. During the course of the experiment the free base as well as the pH shows a gradual decrease. Therefore, even under these conditions, there was apparently some acid formation. It obviously would have taken considerable time for these solutions to reach neutrality. It seemed, therefore, of interest to determine whether solutions of a hexose would undergo interconversion in a buffer solution of neutral or slightly acid reaction.

In a preliminary experiment 25 gm. of *d*-fructose in 100 cc. of water were treated with a solution of 29 gm. of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ in 100 cc. of water which had been made neutral to litmus with phosphoric acid. The clear solution was kept in a thermostat at

⁶ It is interesting that when tetramethylglucose and tetramethylmannose are treated with dilute alkalis, conditions are quite different. Here the interconversion is confined to these two compounds and apparently a true equilibrium is attained ((5) p. 2819).

60° for 48 hours. The solution which had become slightly colored, was evaporated to dryness at reduced pressure and the residue was thoroughly extracted with alcohol. The alcoholic solution, on evaporation at reduced pressure, yielded 15 gm. of gum. On treatment of this with phenylhydrazine below 5° in the usual manner there was obtained a phenylhydrazone which on recrystallization melted at 197–198°, and was apparently *d*-mannose phenylhydrazone. A portion of this in solution with dilute acetic acid was treated with a further amount of phenylhydrazine. From this the bright yellow *d*-mannose phenylosazone, m.p. 203–204°, was obtained. A total of 2.54 per cent of the *d*-fructose has been converted into *d*-mannose under these conditions.

TABLE III.
Effect of Slightly Acid Phosphate Mixture on d-Fructose at 37°.

Days.	Total reducing power as glucose.	Aldoses as glucose.	Mannose.	$[\alpha]_D^{18}$	pH
	gm.	gm.	gm.	degrees	
0	102.7	1.36		–87.8	6.69
27	102.0	6.51	0.098	–78.1	6.64
71	100.1	14.65	2.46	–54.1	6.57
165	97.7	28.00	8.49	–45.8	6.56

A similar experiment with 12.5 gm. of *d*-fructose in 55 gm. of water and 5.75 gm. of anhydrous sodium sulfate yielded no *d*-mannose. Nef⁷ found that solutions of potassium bicarbonate were without effect on hexoses at 20°.

In Table III are given the results of an experiment with 100 gm. of *d*-fructose and a mixture of 74.04 gm. of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ and 9.25 gm. of NaH_2PO_4 in 333.3 gm. of water kept at 37°. The phosphate mixture had a pH of 6.98 before the sugar was added; it then dropped to 6.69 on the addition of the fructose.

From this experiment it is evident that even when the mixture is slightly acid the *d*-fructose in the presence of phosphates is converted into a mixture of the other hexoses.

⁷ Cf. (2) 403, 357, 371.

Glucose.

When *d*-glucose, *d*-fructose, or *d*-mannose is treated in aqueous solution with weak alkalis as, *e.g.* disodium phosphate, or with neutral mixtures of phosphates, there occurs besides the glucose \rightleftharpoons fructose \rightleftharpoons mannose interconversion also the formation of glucose. The question arises whether glucose can be considered as constituting a member of this interconvertible system; *i.e.*, whether the reaction is reversible, so that glucose itself in solution with weak alkalis would yield glucose, fructose, and mannose. One difficulty in the study of this problem has been that glucose has never been obtained in the pure state nor have its properties been accurately described.

Glucose was prepared by Lobry de Bruyn and Van Ekenstein^a by the action of dilute solutions of caustic alkalis, of calcium hydroxide or lead hydroxide on glucose, fructose, or mannose at 100°. The solution becomes distinctly acid and the lead (lead hydroxide gives the best yields) is precipitated with alcohol and tartaric acid. The fermentable sugars, *d*-glucose, *d*-fructose, and *d*-mannose, are then removed from the mixture by fermentation. The glucose, which is obtained as a non-fermentable residual gum, is optically inactive, has been reported as having one-half the reducing power of glucose toward Fehling's solution, shows the ketose reactions of Seliwanoff and Sieben-Dammuller, and molecular weight determinations and elementary analysis correspond to the formula $C_6H_{12}O_6$. Lobry de Bruyn and Van Ekenstein obtained an osazone from glucose, m.p. 165°. The same properties were shown by the glucose obtained by Spoehr and Wilbur ((22) p. 431) on treating glucose, fructose, and invert sugar with disodium phosphate. In view of the incomplete nature of the information concerning this substance and its mode of formation an endeavor was made to gain further evidence of its supposed structure as a ketohexose and of the reactions involved in its formation from *d*-glucose and *d*-fructose.

In an extensive series of experiments on the formation of glucose from hexoses and invert sugar with lead hydroxide and phosphates, involving the preparation of many kilos of glucose, no strictly uniform products were obtained. These experiments will not

^a Cf. (1) 16, 274.

be described in detail. Suffice it to point out that great variations in the yields of non-fermentable residues, in the reducing power of these, and in their aldose content were obtained. From glucose and lead hydroxide the yields of non-fermentable residue ranged from 24 to 41 per cent, the reducing power toward Benedict's reagent was about 51 per cent that of *d*-glucose. The glucose prepared from invert sugar (invertin used for hydrolysis) and lead hydroxide showed a yield of 24 to 35 per cent, a reducing power of 29 to 55 per cent that of *d*-glucose and an aldose content of 11 to 26 per cent. A yield of 15 to 28 per cent of glucose was obtained from invert sugar (from sucrose and invertin) and disodium phosphate; these preparations had a reducing power of 35 to 58 per cent that of *d*-glucose and contained 11 to 18 per cent of aldoses. On the basis of these results it need hardly be emphasized that what has commonly been called glucose is not a homogeneous product. This is due to the fact that the glucose prepared as described contains the products of three different sources of contamination.

First of all, as will be shown later, glucose is itself affected by weak alkalies resulting in the formation of fermentable and presumably also other non-fermentable sugars. Secondly, in the treatment of *d*-glucose, *d*-fructose, *d*-mannose, or invert sugar with alkalies there are always formed varying amounts of saccharinic acids. The amount of these is very decidedly increased at higher temperatures and by the use of stronger alkalies. A third source of contamination is introduced through the fermentation process. We shall turn first to a discussion of this latter aspect of the glucose problem.

Effect of Fermentation on Composition of Glucose Mixture.

That the irregular properties which have been found for different preparations of glucose are in a measure at least due to the fermentation of the sugar mixture was repeatedly found to be the case. An example will serve to demonstrate this.

To a solution of 352.5 gm. of sucrose in 1400 cc. of water were added 2 cc. of an active invertin preparation and the solution kept at 25° for 48 hours; 200 gm. of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ were added and the solution heated in a water bath at 70–75° for 24 hours. The solution was concentrated to 700 cc. by distilling at reduced

pressure, and then by cooling in a salt-ice mixture 120 gm. of sodium phosphate crystallized out and were removed by filtration. The filtrate was diluted to 2800 cc. with 95 per cent alcohol which precipitated most of the remaining sodium phosphate; this was extracted several times with alcohol and all the alcoholic solutions united. These were evaporated to dryness at reduced pressure, dissolved in 3500 cc. of water, and fermented with bakers' yeast for 3 days at 25°. The solution was reduced to one-half its volume by distilling at reduced pressure to remove the alcohol formed; it was then diluted and fermented with a fresh portion of yeast for 3 days. The solution was heated to 80° with a little charcoal for 15 minutes, filtered, and evaporated to dryness at reduced pressure. The residual gum was extracted with alcohol, filtered, and the alcoholic solution evaporated to dryness at reduced pressure. The remaining sugar gum, 196.5 gm. or 52.4 per cent of the original sucrose, showed a reducing power of 81 per cent that of glucose. This sugar gum was subjected to a third fermentation and treated as above, which left 75.6 gm. or 21.8 per cent of the original sucrose with a reducing power of 54.4 per cent that of glucose. A fourth fermentation left 53.6 gm. of sugar gum or 15 per cent the original sucrose with a reducing power of 33.2 per cent that of glucose. Many similar experiments were carried out, all of which demonstrated the decrease in the reducing power of the residual sugar with successive fermentations.

This observation can find an explanation, in part at least, in the fact, known for some time, that in the process of alcoholic fermentation there are formed substances which have a low reducing power or which do not reduce at all (28-32). Just what these substances are has not yet been established. It was thought by us that they might be cycloses. However, separate experiments directed to determine the nature of the non-reducing substances formed in fermentation did not yield any evidence from which the cyclose nature could be concluded.

Experiments on the fermentation of glucose and invert sugar with commercial bakers' yeast (Fleischmann) in which the fermentation was repeated many times by distilling off the water and alcohol and by the addition of fresh water and yeast, showed that a residue with no reducing power or only a very slight one and amounting to about 10 per cent of the original sugar, was obtained.

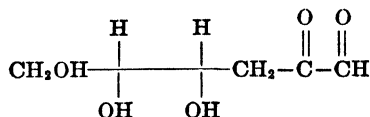
This was not glycerol. Similar results were obtained when the yeast was prepared by the method described by Somogyi (33) and by Raymond and Blanco (34). Thus three cakes of fresh yeast (Fleischmann) were suspended in 150 cc. of water and centrifuged as described by Benedict (35). This preparation was found to be free of starch. It was immediately placed in 600 cc. of a 5 per cent glucose solution and the mixture allowed to stand at 25° for 20 hours. After adding a little charcoal, heating to 60°, and filtering, the solution was evaporated to dryness under reduced pressure. The residue weighed 3.3 gm. and had a reducing power 0.85 per cent that of glucose. A blank with the same amount of yeast, treated in the same manner, left a non-reducing residue weighing 1.7 gm. An experiment with 500 gm. of cane sugar, after nine fermentations, left a residue of 50.8 gm., with a reducing power 3.74 per cent that of glucose.

There is no doubt, therefore, that in the preparation of the glucose a mixture results which contains non-reducing substances. These arise from the fermentation of the fermentable sugars and from the action of the alkali on the sugars, yielding saccharinic acid. Consequently, there is no justification for ascribing to glucose a regular reducing power of one-half the amount shown by glucose.

Effect of Alkalies on the Glucose Mixture.

Benedict, Dakin, and West (36) reported the formation of lactic acid and some unidentified polyhydroxy acids from the action of strong sodium hydroxide solutions on glucose. They were unable to obtain saccharinic acids on treating glucose with an excess of calcium hydroxide "under conditions which would convert glucose into saccharic acid." In this connection it is quite significant that the amount of lactic acid obtained by these workers from glucose is very much less than that obtained by Nef (37) from *d*-galactose, *d*-glucose, *d*-mannose, and *d*-fructose under similar treatment. According to Nef the amount of lactic acid formed is very nearly the same from all hexoses; *i.e.*, 40 to 45 gm. of *dl*-lactic acid from 100 gm. of sugar. If glucose were a 3-ketohexose it could be expected that it would split easily into 2 molecules of glyceraldehyde which under these conditions would rearrange through methylglyoxal to lactic acid. The relatively small amounts of lactic acid obtained

from glucose would seem to indicate that the amount of hexose sugar present was also small; *i.e.*, that the sugar was contaminated with a considerable quantity of non-sugar substances. Moreover, if glucose were an *o,d*-glucosone



as was suggested by Nef, it should in the presence of alkali, easily form saccharinic acid through a benzilic acid rearrangement.

Furthermore, it was important to determine whether glucose, when treated with weak alkalis, is converted into other hexose sugars and whether among these there is *d*-glucose, *d*-mannose, or *d*-fructose. To a solution of 18.5 gm. of glucose in 74.3 gm. of water were added 21.2 gm. of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ and 3 cc. of xylene; the solution was tightly stoppered and kept in a thermostat at 37°. The glucose had been prepared from invert sugar and disodium phosphate and had a reducing power equal to 40.6 per cent that of glucose. After 98 days the disodium phosphate was crystallized out in the cold, the solution evaporated at reduced pressure, and the gum extracted with alcohol. After evaporation of the alcohol the gum, 15.3 gm., was dissolved in water and a portion of this solution was placed in a fermentation tube with a preparation of washed bakers' yeast (Somogyi-Benedict) and kept in a thermostat at 25°. After 3 hours considerable carbon dioxide had been formed, which was not increased appreciably with time. From the amount of carbon dioxide formed it was calculated that the total quantity of fermentable hexose sugar was 7.5 per cent of the quantity of glucose which had been recovered from the phosphate treatment. Duplicate determinations gave the same results. The original preparation of glucose, subjected to the fermentation test with the same preparation of yeast, developed no gas at all. But when a small quantity of *d*-glucose was added to this, active fermentation took place, indicating that the yeast was capable of producing fermentation.

Benedict, Dakin, and West demonstrated the formation of methylglyoxal from glucose by means of the method of Neuberg and Oertel and that of Windaus and we have confirmed this observation.

Glucose Phenyllosazone.

In the earlier work on glucose the fact was emphasized that this forms a phenyllosazone of constant melting point and definite composition, corresponding to a hexose phenyllosazone. In our own experiments it was repeatedly found that glucose forms a phenyllosazone very easily, which when recrystallized from alcohol and water melts at 165–170°, gives analytical values agreeing with a hexose phenyllosazone, and corresponds to the glucose phenyllosazone described by Lobry de Bruyn and Van Ekenstein. However, when this phenyllosazone was extracted and recrystallized from different solvents, it was found possible to resolve it into several fractions, each having a different melting point and having a nitrogen content corresponding to hexose phenyllosazone. This indicated that glucose is not a homogeneous substance but is rather a mixture of a number of hexose sugars. Several preparations of glucose, prepared by the action of lead hydroxide and of disodium phosphate on glucose and on invert sugar, as has been already described, were converted into the phenyllosazones and these were resolved.

In general, glucose phenyllosazone was found to contain a high melting fraction (195–207°) which was only slightly soluble in water, alcohol, benzene, and ether and moderately soluble in pyridine; a fraction melting around 190°, moderately soluble in alcohol, slightly soluble in water, benzene, and ether, and very soluble in pyridine; a fraction melting at 160–170°, soluble in alcohol, moderately soluble in benzene, slightly soluble in ether and water, and very soluble in pyridine; a fraction melting around 150°, very soluble in alcohol, benzene, ether, moderately soluble in water, and extremely soluble in pyridine. The resolution into these fractions was obtained by drying the crude crystals of glucose phenyllosazone in a vacuum desiccator, then extracting with cold benzene to remove the tar, and extracting the residual crystals with boiling 95 per cent alcohol which left the high melting fraction as a residue. Upon cooling the alcoholic solution the next high melting fraction crystallized out. The filtrate from this fraction usually formed another crop of crystals on diluting with benzene. After filtering and evaporating the filtrate to dryness under reduced pressure, the residue was dissolved in alcohol,

decolorized with charcoal, and recrystallized by the addition of water while hot, thus giving the low melting fraction. These various fractions were recrystallized and finally analyzed for percentage composition of nitrogen. The phenylosazones obtained from glucose which had been prepared with disodium phosphate had a nitrogen content corresponding to hexose phenylosazone (15.64 per cent), the extremes ranging from 15.32 to 15.68 per cent of nitrogen. The lower melting phenylosazones obtained from glucose which had been prepared from lead hydroxide did not give good analyses; the nitrogen content was usually low (13 to 14 per cent) and this was not materially affected by repeated recrystallization.

Oxidation of Glucose with Bromine.

Several attempts were made to determine the nature of glucose on the basis of its behavior toward bromine and iodine. Experience has taught that ketoses are not oxidized by iodine in the presence of sodium carbonate. However, if glucose is a ketohexose, it differs from all known ketohexoses in that it reduces iodine about 0.25 to 0.5 times the value shown by glucose, the reducing power varying with different preparations of glucose. That this action of iodine on glucose is not due to the presence of the residue left by the fermentation process is proved by the following fact. In a separate experiment the residue from 500 gm. of invert sugar after eight fermentations amounted to 56.4 gm. This residue showed a total reducing power toward Benedict's solution of 0.52 per cent that of glucose and a reducing power toward iodine of 0.77 per cent that of glucose.

A number of glucose preparations in water solution were treated with bromine in the presence of calcium carbonate or lead carbonate. An endeavor was made to identify the acids thus formed. It was, however, impossible to obtain any metallic or alkaloid salts from these acids in a sufficiently pure state to warrant any definite conclusions regarding their composition. The reducing power of the glucose preparations was decidedly decreased after successive treatments with bromine. In the glucose prepared by means of disodium phosphate this decrease approximated the amount of aldohexose originally present in the glucose as shown by iodine titrations. In the glucose prepared by means of lead hydroxide

successive treatments with bromine caused a continual decrease in the reducing power of the sugar, apparently bearing no relation to the values obtained for the iodine titrations of these original glucose preparations.

The results of the resolution of glucose phenylosazone and of the oxidation of glucose with bromine indicate quite clearly that glucose is not a single compound but is rather a mixture of ald- and ketohexoses.

Glutoheptonic Acid.

An endeavor to gain some further evidence relating to the structure of glucose was made by converting this into the corresponding heptonic acid by means of the cyanohydrin reaction, as has been done in the determination of the position of the carbonyl group in other hexose sugars. The method employed for the preparation of the heptonic acid was that described by Fischer (38). As was found by Kiliani (39) to be the case with the fructose heptonic acid, neither the barium nor calcium salts of the glutoheptonic acid could be obtained in crystalline form. However, the latter compound did form a basic calcium salt of low solubility which was used to separate the acid from impurities.

The basic salt was prepared in the following manner. After the solution containing the glucose, hydrocyanic acid, and ammonia had stood in the dark, at room temperature, for 5 days, it was heated on the water bath to expel the excess of hydrocyanic acid. The solution was then treated with freshly prepared calcium hydroxide in small amounts and the heating continued for 4 hours. The insoluble basic salt was filtered and more calcium hydroxide added to the filtrate and heated and a second crop of basic calcium salts was thus obtained. The combined basic calcium salts, in suspension in water, were made faintly acid with a solution of oxalic acid, heated, and filtered. The filtrate, after being decolorized with charcoal, was again treated with calcium hydroxide and the basic calcium salts separated by filtration. This process was repeated three times. These basic calcium salts are not pure, as is shown by the analyses, probably due to occluded calcium hydroxide. 0.3366 gm. of basic calcium salt, on ignition, yielded 0.0765 gm. of CaO or 16.27 per cent calcium. Calculated for $C_7H_{13}O_8CaOH$, 14.18 per cent calcium.

The normal calcium salt was prepared from the basic salt by neutralizing the latter, in water suspension, with a solution of oxalic acid, filtering, concentrating the filtrate to a small volume, and precipitating the calcium salts from this with methyl or ethyl alcohol. The precipitated salts were filtered, again dissolved in water, and reprecipitated; this procedure was repeated several times. In this manner a white, crystalline salt was obtained. It was not possible to crystallize the salt in any other way. On analysis, the calcium percentage of the salt obtained in different experiments was 8.49, 8.22, 7.89, and 7.82, the theory for $(C_7H_{13}O_8)_2Ca$ being 8.17 per cent calcium. This calcium salt showed no optical rotation. Attempts to obtain a crystalline lactone from this salt were not successful.

Reduction of Glutoheptonic Acid.

28.5 gm. of calcium glutoheptonate were dissolved in 280 gm. of hydriodic acid (sp. gr. 1.70), 11 gm. of red phosphorus added, and heated in an oil bath, under a reflux, at 150–170° for 7 hours. The mixture was diluted with water, extracted with ether, and treated with a little mercury to remove traces of iodine. The filtered ether solution was evaporated and the residual oil was allowed to stand with zinc and dilute sulfuric acid for 12 hours. This was again extracted with ether and the extract distilled with steam; the distillate was extracted with ether, from which a residual oil, 5 gm., was obtained. The oil was carefully neutralized with lime water and concentrated to about 50 cc. in a vacuum desiccator over sulfuric acid, when crystals of the calcium salt of heptylic acid separated out. On analysis 0.2007 gm. of this calcium salt yielded 0.0931 gm. of $CaSO_4$, or 13.65 per cent of calcium. Calculated for $(C_7H_{13}O_2)_2Ca$, 13.42 per cent calcium.

The position of the carbonyl group in the original glucose would determine which of the isomeric heptylic acids was formed. That is, a straight chain aldohexose would yield normal heptylic acid, a 2-ketohexose would yield 2-methylhexylic acid (methyl *n*-butyl acetic acid), and a 3-ketohexose would yield α -ethyl *n*-valeric acid (ethyl propyl acetic acid). In order to identify the heptylic acid obtained, it was converted into the amide by first preparing the acid chloride with phosphorus trichloride and dropping the acid chloride into an ice-cold solution of ammonium hydroxide (40).

From this the amide separated out in white crystals which were recrystallized from water and alcohol. The amide thus prepared melted at 68–70° corrected (Berl block); in another experiment an amide melting at 67–68° was obtained. The *n*-heptamide (corresponding to aldohexose) has a m.p. of 94–95°, 2-methyl hexamide (corresponding to 2-ketohexose) a m.p. of 70–72.5°, and α -ethyl valeramide (corresponding to 3-ketohexose) a m.p. of 102.5–103.5°. It is to be concluded, therefore, that the heptylic acid obtained from glucose was the 2-methyl hexylic acid.

From the evidence of these experiments it may be concluded that glucose, prepared either by means of lead hydroxide or disodium phosphate, is not a homogeneous substance, but that there are present, besides some saccharinic acids and by-products of fermentation, both aldo- and ketohexoses; no evidence of the presence of a 3-ketohexose or an *o,d*-glucosone was obtained. The cyanohydrin reaction and subsequent reduction of the heptonic acid do give evidence of the presence of a 2-ketohexose.

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THE NATURE AND BIOLOGICAL AVAILABILITY OF ALMOND CARBOHYDRATES.

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Although a number of studies of the biological value and chemical properties of nut proteins and oils have been reported, very little has been published as to the nature and availability of the carbohydrate fractions of the oily nuts. Digestibility studies upon these carbohydrates, moreover, are almost entirely lacking. The usefulness of such nuts as food for diabetics is often suggested, and the development of nut flours for the making of bread and cake substitutes occasionally recommended, but active use of these palatable foods has been delayed by uncertainty as to their digestibility. Lack of digestibility or presence of unabsorbable fecal residue may be a characteristic of certain nut carbohydrates and therefore a property of value in the recommendation of nuts in the treatment of constipation.

Certain experiments (1) upon rats with washed, fat-free almond meal in this laboratory some years ago led to the surmise that almond carbohydrates may contain a bulky unabsorbable fraction which may materially affect the nutritive value of this part of the nut as well as add to its anticonstipation effect. This study of both the chemical character and the biological availability of almond carbohydrates was therefore undertaken.

The actual nature of the reserve carbohydrates of seeds has been determined in only a few cases, and for the edible nuts only two or three reports can be cited. The variety of substances which may and do occur in the nitrogen-free extract of all foods is probably great, including as it does sugars, starch, dextrins, gums, organic acids, lignin, tannins, pentosans, alcohols, alkaloids, cellulose, and the so called hemicelluloses, such as mannans,

galactans, and inulin. Since these substances are of widely differing chemical properties and nutritive value, it is obvious that significance can be attached to analyses of this group only by fractionation into its constituents. Early in the development of food analysis a number of investigators, such as Atwater (2), Stone (3), Tollens (4), and Sherman (5) suggested methods by which more information might be obtained about these substances, but surprisingly few such studies have been made in the quarter century since these suggestions were made. A few examples of such analyses are those of Street and Bailey (6) on the carbohydrates of soy beans, Petersen and Churchill (7) on the navy bean, and Neale (8) on peanut meal. There are such decided differences as to digestibility and availability among the carbohydrates found in vegetable foods that even the simplest analysis into sugars, dextrins, starch, pentosans, and other hemicelluloses and crude fiber is of real importance to those interested in the nutritive value of foodstuffs.

Among the fatty nuts such as the almond, walnut, pecan, filbert, and Brazil nut, starch is usually said to be absent, crude fiber is usually not reported, and although the total carbohydrate is close to 15 per cent, very little further information about its make-up is available.

Certain studies of such plant carbohydrates, however, have been made by investigators primarily interested in the functions of these substances in the vegetable organism. Among these studies which are reviewed and quoted by Czapek (9), are certain valuable contributions chiefly by Schulze and his coworkers upon the carbohydrates of nuts, including almonds. Their results are later discussed and compared with those here reported.

It was evident from proximate analyses made upon two varieties of almonds of different crops that considerable variation may occur. Thus the composition of Nonpareil almonds of the 1923 California crop may be compared with seedlings of the 1925 crop and with the Atwater and Bryant average as shown in Table I. The total carbohydrate of both the lots used in this investigation is lower than that usually reported and the fat and protein correspondingly higher. The nuts used for our analyses were blanched, however, and this fact no doubt partly accounts for the low carbohydrate percentage. Since the fat-free meal was

to be made from blanched almonds, it seemed best to obtain figures for proximate analysis on the same nuts.

The almonds used in the work here reported were sheller-run seedlings and Nonpareils of the 1925 crop.¹ The nuts were blanched, dried, ground, and most of the fat removed by pressure followed by extraction with boiling anhydrous ether. The resulting fine white meal was submitted to the usual proximate analysis, the results of which are also shown in Table I. This meal, con-

TABLE I.

Proximate Composition of Almonds and Almond Meal.

Figures are expressed in per cent.

	Protein.	Fat	Ash.	Water.	Total carbohydrate (N-free extract).	Crude fiber.	Carbohydrates not crude fiber.
Almonds.							
Nonpareil (blanched) 1923 crop.*	23.0	64.0	0.5	5.0	7.5	1.2	6.3
Seedling (blanched) 1925 crop.*	29.0	56.0	1.5	5.0	8.5	2.7	5.8
Atwater and Bryant (10).....	21.0	54.9	2.0	4.8	17.3	2.0	15.3
Jaffa (11).....	21.4	54.4	2.5	4.9	16.8	5.0	13.8
Langley (12).....	23.1	53.1	2.5	7.3	11.2	2.8	8.4
Almond meal.							
Sample 1 (3 analyses).....	46.6	4.1	6.3	11.8	31.2	4.0	27.2
“ 2 (4 “ “).....	49.3	4.5	6.5	9.7	30.0	4.4	25.6
Average	47.9	4.3	6.4	10.7	30.6	4.2	26.4

* Analyses were made by Vera MacNair.

taining 30.6 per cent of carbohydrates, was then used for the separation and identification of its carbohydrates.

Methods.

1. *Total Reducing Sugars from Direct Acid Hydrolysis.*—Direct hydrolysis of the meal by 3 per cent hydrochloric acid was carried out by boiling under the reflux condenser for 2½

¹ The almonds were given us for this purpose by the California Almond Growers' Exchange.

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hours. After neutralization, the reducing sugars in an aliquot were determined by Bertrand's copper reduction and permanganate titration method. The resulting figure, expressed as glucose, represents total reducing sugars, but such hydrolysis for comparison with animal availability studies may not be satisfactory since pentoses might be included, or some of the maltose destroyed, as shown by Davis and Daish (13).

TABLE II.

Carbohydrate Fractionation by Alcohol Extraction and Acid Hydrolysis of Almond Meal.

Sample No. and material.	Treatment.	No. of determinations.	Reducing sugar yield, per cent of meal.	Nature of carbohydrate.
1. Dry fat-free meal.	Extraction with boiling 95 per cent alcohol, extract.	5	0.49	Preformed reducing sugars.
2. 95 per cent alcohol extract.	Acid hydrolysis.	11	6.33	Soluble reducing and condensed sugars.
3. Sample 7 - 5.		2	7.57	" "
4. Dry fat-free meal.	Extraction with 10 per cent alcohol.			
5. Residue from 10 per cent alcohol extraction.	Acid hydrolysis.	2	7.06	Hydrolyzable polysaccharides.
6. Sample 7 - 2.			8.30	" "
7. Dry fat-free meal.	Direct hydrolysis with HCl.	4	14.63	Total acid-hydrolyzable carbohydrates.

2. *Reducing Sugars Produced by Hydrolysis of Polysaccharides.*—The reducing sugars were determined by extracting 5 gm. portions of the meal with 150 cc. of 10 per cent alcohol on a hardened filter paper, hydrolyzing the insoluble residue, and determining reducing sugars by Bertrand's method. This is a modification of the preparation suggested by Cake and Bartlett (14).

3. *Soluble Reducing Sugars, Free and Condensed.*—The difference between total reducing sugars obtained after acid hydrolysis

and that obtained on hydrolysis of polysaccharides, represents the free sugars. This was checked by direct hydrolysis of a 95 per cent alcohol extract, with good agreement (see Samples 2 and 3, Table II).

4. *Starch, and Other Taka-Diastase-Hydrolyzable Carbohydrates.*

—The method of Olmsted (15) was followed, taka-diastase being used because of the frequent criticisms directed against the acid hydrolysis method. Although the qualitative test of the meal for starch with iodine was negative, the diastase apparently hydrolyzed certain substances possibly dextrins and maltose, or certain hemicelluloses, so that a starch fraction was separated. Schulze and Castoro (16) have shown that hemicelluloses may be slowly hydrolyzed by diastase, and that the oily seeds yield small amounts of reducing sugar after treatment with malt extract.

Samples of the meal, 5 or 10 gm., were heated on the boiling water bath for an hour with 150 cc. of water, cooled, and 0.1 gm. of dried taka-diastase added. The mixture was incubated at 37° for 17 hours with a few drops of toluene or xylene as preservative. It was then filtered, made up to volume, treated with lead acetate, refiltered, and reducing sugar determined in the filtrate before and after hydrolysis with acid. Control determinations were made on the taka-diastase alone as well as upon the reagents. The permanganate used in the Bertrand titration in this and the other determinations of reducing sugar was frequently standardized against solutions of pure anhydrous glucose.

The figures obtained on this filtrate before hydrolysis represent free reducing sugars, dextrins, and starch, but presumably not sucrose and most hemicelluloses. After hydrolysis, the contents are the same but soluble disaccharides and polysaccharides are added. Checks were obtained also by determination of reducing sugar in the boiling water extract of the meal after treatment with lead acetate and previous to the addition of the taka-diastase, both before and after acid hydrolysis. These figures should give the amounts of water-soluble preformed and hydrolyzable reducing sugars, and the difference between the latter and that found after taka-diastase digestion should represent starch and dextrins. These figures are shown in Table III.

5. *Pentoses and Pentosans.*—The pentoses and pentosans were determined by the method of Youngburg (17). This is a micro

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TABLE III.

Carbohydrate Fractionation by Water Extraction and Diastase Hydrolysis of Almond Meal.

Sample No. and material.	Treatment.	No. of determinations.	Reducing sugar yield, per cent of meal.	Nature of carbohydrate.
1. Dry fat-free meal.	Hot water extraction, extract.	12	0.70 (as glucose).	Preformed reducing sugars.
2. Hot water extract.	Acid hydrolysis.	5	9.17 (as invert sugar).	Free reducing sugars, soluble condensed sugars.
3. Sample 2 - 1.			8.47	Soluble condensed sugars, probably largely sucrose.
4. Meal, boiled with water.	Incubated with taka-diastase, filtrate.	22	3.85 (as glucose).	Free reducing sugars, dextrans, starch.
5. Take-diastase filtrate.	Acid hydrolysis.	13	10.74 (as glucose).	Free reducing sugars, dextrans, starch, soluble condensed sugars.
6. Sample 5 - 2.			1.57	Dextrins and starch, possibly some hemicelluloses.
7. Sample 10 - 5.			3.89	Hydrolyzable polysaccharides other than starch.
8. Dry fat-free meal.	Furfural distillation after acid hydrolysis.	16	7.56 (as arabinose).	Pentoses and pentosans.
9. " "	Crude fiber determination.	9	4.20	Chiefly cellulose.
10. " "	Direct acid hydrolysis.	4	14.63 (as glucose).	Sugars and all acid-hydrolyzable polysaccharides.

method in which furfural is produced from the pentoses by hydrolysis with phosphoric acid followed by steam distillation, and determined in the distillate by a colorimetric method involving the use of aniline acetate. Recovery of pure pentoses by this method is entirely satisfactory only for *d*-xylose, and other non-pentose substances, particularly maltose, dextrose, dextrin, and sucrose, respond to the extent of 3 to 5 per cent to this test.

TABLE IV.
Summary of Distribution of Almond Carbohydrates.

Sample No. and fraction.	Per cent. of meal.	Per cent of meal carbo- hydrates.	Per cent of almond (blanched).
1. Total carbohydrate of fat-free almond meal, by difference.	30.60	100	8.5
2. Free reducing sugars.	0.70	2.2	0.2
3. Sucrose and other easily hydrolyzable soluble sugars.	8.47	27.6	2.3
4. Starch, dextrins, and other diastase-hydrolyzable saccharides.	1.57	5.1	0.4
5. Acid-hydrolyzable polysaccharides not including starch.	3.02	9.8	0.8
6. Pentoses and pentosans.	7.56*	24.7	2.1
7. Crude fiber.	4.20	13.7	1.2
Total available carbohydrates.	14.63	47.8	4.0
Sum of percentages, Samples 2, 3, 4, 5, 7.	17.86	58.4	4.9
Undetermined.	12.74	41.6	3.6

* 3 to 5 per cent of soluble sugars may be included in this figure which therefore may represent 0.46 per cent of meal, or 1.5 per cent of meal carbohydrates. Most of this fraction is expressed also in the acid-hydrolyzable figure, 14.63, and is therefore not included in the sum.

The results, calculated as arabinose, since this sugar has been shown to occur in the almond, nevertheless appear more satisfactory than those obtained by the phloroglucide method. Pentosans were determined both in the meal and in the residue from alcohol extraction.

6. *Crude Fiber*.—Crude fiber, which is possibly largely cellulose, was determined in the meal by the official method (18). The results of this method of analysis of the carbohydrates are given in Table III.

Another scheme of separation following that of Street and Bailey (6) on soy beans was also used. Samples of the fat-free meal were boiled with 95 per cent alcohol for 18 hours, filtered, the alcohol distilled off, the residue made up to 100 cc. with water, filtered again to remove water-insoluble substances, again made up to volume, and reducing sugar determined as usual before and after acid hydrolysis. By this treatment probably all sugars were extracted including dextrose, levulose, maltose, sucrose, and, if present, probably free pentoses, soluble pentosans, and raffinose. The figures obtained are shown in Table II.

As indicated by the summary in Table IV, 58.4 per cent of the almond meal carbohydrate was accounted for by the methods described, 47.8 per cent being acid-hydrolyzable and therefore presumably digestible. The largest fraction detected was made up of water-soluble condensed sugars, probably sucrose, which constituted 8.47 per cent of the whole meal, or 27.6 per cent of the nitrogen-free extract.

The proportion of pentoses yielded on phosphoric acid hydrolysis is 7.56 per cent of the meal, a figure which is probably too high, since other substances present besides pentoses may yield furfural. This figure also overlaps that for reducing sugars obtained by direct acid hydrolysis of the meal, and is therefore not included in the summation of percentages in Table IV, although it is by no means certain that all pentosans are hydrolyzed by the procedure used. It is possible that some of the furfural obtained represents pentosans not elsewhere broken down. The amount of additional carbohydrate accounted for if this were true, would be 24.7 per cent, making the known total 83.1 per cent.

The crude fiber results were unusually consistent, and their values similar to those hitherto reported for almonds. If this may be reckoned as carbohydrate, probably cellulose, it represents the next largest fraction after sucrose and pentosans, 4.2 per cent of the meal, and 13.7 per cent of the nitrogen-free extract.

The undetermined material, representing 41.6 to 16.9 per cent of the carbohydrate may be of the hemicellulose type, possibly mannans, but may also be entirely non-carbohydrate in nature. The latter possibility seems more likely to us.

In the long and important series of studies upon the carbohydrates of seeds made by Schulze and his coworkers ((9) p. 420)

almond kernels and shells were included, particularly in the work by Schulze and Godet (19). Much of their analysis was qualitative only, but included the necessary and difficult task of the isolation and identification of certain products such as galactose, glucose, arabinose, and mannose. Their analysis of almond carbohydrates may be summed up as follows: Sucrose, galactose, and arabinose were present either free or condensed, but there is no mannose or fructose; 2.51 per cent of dry almond was present as hemicelluloses, probably chiefly pentosans, which constituted 2.38 per cent of the dry shelled nut. Cellulose was shown to be present.

Vallée (20) found 2.97 per cent of sucrose and 0.09 per cent of reducing sugar in sweet almonds. Langley (12) reported for the Chinese almond, sucrose 2.1 per cent, crude fiber 3.17 per cent, and pentosans 3.8 per cent, thus accounting for 9 per cent of the 15 per cent of carbohydrates by difference.

Our results, as shown in Table IV, differ from those of Schulze and Godet chiefly in our detection of small amounts of free reducing sugars and of diastase-digestible carbohydrate. The quantity of sucrose is high and of pentosans low as compared with those reported by both Langley and Schulze and Godet, in view of our low total carbohydrate. On the whole, however, the agreement is good, if the differences in procedure and in sample involved are considered.

Qualitative Tests.

Results of the mucic acid test obtained by oxidation of the hydrolyzed meal with strong nitric acid were very slight. An attempt was made to determine galactans by the official method (18), but the resulting figure, 0.27 per cent, seems much lower than might be expected. However, the difference between total hemicellulose and pentosans as found by Schulze and Godet, is of a similar order, 0.13 per cent, and the fact may be that galactans form an inconsiderable portion of the hemicelluloses of the almond.

A strongly positive Seliawanoff test for fructose was obtained both on the meal and in the water extracts, thus confirming the presence of sucrose.

Phenylhydrazine acetate was added to the cool neutral solutions of hydrolyzed and clarified meal filtrates. No insoluble

mannose phenylhydrazone was obtained, but on further treatment with excess of the reagent phenylglucosazone, identified by its melting point of 208–213°, was obtained. This meant only that glucose, fructose, or mannose was present.

The carbohydrate of the almond may thus be seen to be about 48 per cent digestible by dilute hydrochloric acid, and to consist of sucrose one-third, cellulose one-seventh, pentosans one-fourth, and the undetermined remainder, about 40 per cent, probably of difficultly hydrolyzable hemicelluloses, or alkaloids, acids, tannins, or other non-carbohydrate substances. About one-third of the material usually reported as carbohydrate in this nut may therefore be expected to be available in the animal body.

Biological Availability.

Digestibility studies upon nut carbohydrates are few in number and these usually are included in feeding trials with practically mixed diets. Thus, Jaffa (11) reported 94 per cent digestibility for total carbohydrate in six experiments in which he used various fruits along with almonds, the nuts furnishing only 5 to 25 per cent of the carbohydrate eaten. Cajori (21) in 1918 mentioned a technique for determining the digestibility of nut carbohydrates and stated that "in general the proteins (*i.e.* nitrogenous components) and carbohydrates of the nuts studied were absorbed in large part; and in no case does the quantity of nitrogen or carbohydrate appearing in the feces indicate that these nuts are especially resistant to the digestive functions of the alimentary canal." No data as to the carbohydrates are given however, although it was stated that quantitative work on dogs and men had been performed. The raw almond was said to be as fully digested in these experiments as the steamed or roasted nut.

It might be assumed from the foregoing investigation of the nature of the almond carbohydrates that only about one-third would be available in the animal body, and that the remainder would prove largely unabsorbable, accounting for the bulky white feces excreted by animals fed large quantities of the meal. Several methods of testing carbohydrates for biological availability are in use, but none may be said to be thoroughly standardized and reliable. The simple determination of carbohydrate in food and in feces over definite feeding periods cannot be used

for this purpose because of the rapid and variable bacterial decomposition of unabsorbed carbohydrates in the intestine. Even crude fiber is noticeably attacked under these conditions. Carbohydrate utilization studies are therefore far less simple and fewer in number than similar studies of protein, or of calcium, phosphorus, and other ash elements. In the extensive investigations of Rubner (22) upon utilization of various foods, particularly breads, during the recent war he used the device of determining total caloric value of food and of resulting feces, as well as total nitrogen, pentosans, starch, and cellulose. The disappearance of pentosans and cellulose varied considerably and without apparent reason. Thus in a white bread experiment, 93 per cent of pentosans and 78 per cent of cellulose disappeared, but with whole wheat bread only 66 per cent and 1.5 per cent disappeared.

Instead of such direct determination upon feces more reliable methods appear to consist of observation of effects of carbohydrate ingestion upon respiratory quotient, prevention of acidosis, increase in urinary sugar of phlorhizinized animals, protein-sparing effect, rise in level of blood sugar, and glycogen formation. Thus Hoffman (23) found that the hemicellulose of cabbage disappears from the intestine of the rabbit but does not increase the sugar in the urine of the phlorhizinized animal. Similarly Lusk (24) tested the cellulose of cauliflower by means of phlorhizinized dogs, and Lewis and Frankel (25) inulin, in both cases with negative results. Olmsted (15) compared the sugar yield of certain vegetables when fed to phlorhizinized dogs with similar yield on taka-diastrase hydrolysis.

Rose (26) fed salep mannan to diabetic patients and noted no sugar excretion and persistence of ketosis as indications of mannan digestion. She also failed to obtain glycogen production in rabbits fed this mannan, as did Neuberg and Mayer (27). In all these cases there was but little indication of the availability of sugars from the hemicelluloses in metabolism. Schwartz (28) states that it is impossible to treat of the digestibility of these substances as a class, but that each type must receive consideration separately and that distinction must be drawn between soluble and insoluble forms.

Bodey, Lewis, and Huber (29) compared the glycogen content of the livers of rats fed fructose or inulin with those of similar

animals fed butter oil, and found a rise in the glycogen of the inulin-fed rats. Cori and Cori (30) in a long series of experiments upon normal and insulinized rats have recorded respiratory quotients, body and liver glycogen, blood sugar, and heat production after varying periods of sugar absorption. They have been able to establish the rate of absorption of the various sugars, their value as glycogen formers, and the changes produced by insulin in distribution of absorbed sugar between glycogen formation and oxidation. In our work upon almond meal we followed chiefly the glycogen detection scheme of Cori and Cori, although we tried also to obtain urinary sugar figures for almond-fed rats on the supposition that these might be raised above normal by the excretion, as suggested by Folin and Berglund (31), of "a motley variety of carbohydrate products and carbohydrate derivatives including di- and polysaccharides."

Methods Used in Availability Tests.

Rats were fed almond meal and control diets of composition as follows:

<i>Almond Diet.</i>		<i>Control Diet.</i>	
	<i>per cent</i>		<i>per cent</i>
Almond meal.....	70	Casein	38
" oil	30	Corn-starch	24
		Salt mixture (Osborne and	
Yielding:	<i>per cent</i>	Mendel)*..	4 5
Protein.....	33 5	Almond oil	33
Fat.....	33 0		
Carbohydrate	21 4		
Ash.....	4 4		
Water.....	7.5		
Yeast 0.5 gm. and cod liver oil, 3		Yeast and cod liver oil as for	
drops, daily per rat separately.		almond diet.	

* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).

Some difficulty was encountered in incorporating such a large proportion of the oil into the control diet. The oil separated from the other ingredients largely on standing, and at first the rats refused the mixture. The defatted almond meal, however, quickly and effectively absorbed the oil, making an apparently dry non-greasy preparation. The physical property of the

almond proteins and carbohydrates, which allows the incorporation of such enormous quantities of oil, is very striking and worthy of investigation.

After the rats had been fed the diets a week or more, food intake and fecal output records were made for several periods of 2 weeks each. The feces were weighed, dried at 70°, weighed again, and analyzed for total sugar, crude fiber, and pentosans. The results

TABLE V.
Effect of Almond Meal upon Composition of Feces.

Rat No.	Diet.	Length of period	Food intake per wk.	Feces.		Pentose.		Crude fiber.	
				Fresh weight per wk.	Dry weight per wk.	In food per 2 wks.	In feces per 2 wks.	In food per 2 wks.	In feces per 2 wks.
		wks.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
51	Control.	4	75	6.1	5.8		0.16		
							0.36		
48	"	4	68	4.9	4.2		0.04		
							0.04		
61	Almond.	4	81	18.1	12.4	9.2	0.73	4.8	1.84
						10.1	1.08	5.2	2.67
65	"	4	78	18.9	11.7	7.2	2.10	4.2	2.29
						7.3	1.70	4.4	2.00
4	"	4	69	9.4	8.4	8.6	0.13	4.5	0.92
						7.8	0.10	4.1	0.48
3	"	4	77	9.5	8.5	7.0	0.32	4.2	0.07
						8.3	0.23	4.4	0.07
Average.	Control.	4	73	5.5	5.0		0.15		
"	Almond.	4	76	14.0	10.5	9.4	0.80	4.4	1.2

are shown in Table V. No sugar was found in any case before hydrolysis in the feces. The bulk of fresh feces excreted by the almond-fed rats was nearly 3 times that of the control animals and contained twice as much dry matter. The effectiveness of the almonds as a laxative has been noted in several experiments (32) with human subjects previously carried on in this laboratory. An attempt was made at one time to use a phlorhizinized dog for testing the sugar-forming capacity for almond meal, but the laxative effect was so great as to interfere with the test. One or

two digestibility studies upon two young women were likewise brought to an end by a similar effect.

The pentosans and crude fiber of the almond diet largely disappeared from the intestinal tract of the rats as shown in Table V, and particularly in the case of Rats 3 and 4 which were much older animals than the others. These results do not necessarily indicate, however, that the carbohydrates thus disappearing have become available in the rats' metabolism. They may be accounted for by destruction by intestinal bacteria without absorption.

Glycogen Formation.

In order to discover what proportion of the almond carbohydrate is of value in glycogen production, two series of rats were fed the almond and control diets respectively for 24 hours following 24 hours of fasting. All rats had been kept upon the corresponding diets for a week or more before the fast. The animals were killed with chloroform, livers and intestinal tracts at once removed, and glycogen determined according to Pflüger's method. In several cases the bodies were also immediately ground, body glycogen determined as suggested by Cori and Cori (33), and blood sugar determined by the method of Folin and Wu. The latter method was also used for sugar produced by the hydrolyzed glycogen. The amount of acid used in hydrolyzing the glycogen was reduced to 12.5 cc. of hydrochloric acid (sp. gr. 1.19) per 500 cc. of filtrate, since in an earlier series most of the liver glycogen was destroyed by the larger amount suggested in the original method.

An attempt was made to determine urinary sugar also but this was not entirely successful because of food scattering. In another experiment with two human subjects, however, it was found that the urinary sugar did not vary from the normal during a preliminary period on a mixed diet and an almond meal period. The range observed was 419 to 561 mg. per day. In view of the results obtained by Greenwald, Gross, and Samet (34) as well as by Folin and Berglund (31) and many others it is somewhat surprising that a pentose-rich substance such as the almond meal should produce no perceptible increase in urinary sugar.

In our rat determinations however, the control animals excreted

3 to 5 mg. of reducing sugar daily, which was increased to 10 to 18 mg. after hydrolysis. The almond-fed rats excreted 4 to 5 mg. of reducing sugar, which was increased to 25 to 29 mg. after hydrolysis. This indicates the excretion of about twice the amount of probably unusable polysaccharides resulting from the almond diet as compared with that excreted by the controls.

The blood sugar of the control rats was distinctly higher than that of the almond-fed and the fasted rats, as shown in Table VI. The differences are large enough to leave little doubt of the reality of this distinction, 173 ± 6 mg. per 100 cc. in the control series, 123 in the fasting rats, and 128 ± 4 in the almond-fed animals. This blood sugar level in the fasted rats is higher than that reported by Cori and Cori (33), 97 mg. per 100 cc., but the control series shows the same level as their glucose-fed animals.

Liver glycogen of the almond-fed rats was likewise less than that of the control series, 0.43 ± 0.046 per cent of the liver weight, as compared with 1.18 ± 0.069 per cent. The variability of both blood sugar and liver glycogen was considerably greater among the controls than among the almond-fed rats, standard deviations for the control series being 28 mg. and 0.32 per cent, and for the almond-fed series 14 mg. and 0.13 per cent.

Body glycogen was similarly less in the almond-fed rats, 0.026 per cent of body weight as compared with 0.074 per cent in the controls. The number of determinations in the latter series is so small, however, that confident comparisons cannot be made. The body glycogen of the four fasted rats was very small, 0.015 per cent of body weight, a figure which is much smaller than that found by Cori and Cori (33), who reported 0.114 per cent for rats fasted 48 hours and 0.134 to 0.150 per cent for those fasted 24 hours. All of the body glycogen figures obtained in these experiments are low compared with those of Cori and Cori, probably for the reason that the control rats had practically fasted for a week or more preceding the test since they refused to eat the oily control diet. However, they ate the diet satisfactorily for at least 1 or 2 days before the final fasting and feeding days were begun. This may account for the low body glycogen and high liver glycogen figures found. Muscle glycogen has been found to be a possible source of liver glycogen even though according to Mann and Magath (35) it is not a direct source of blood sugar.

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Role of Almond Carbohydrates As Glycogen Formers (When Fed 24 Hours, after a Fast of 24 Hours).

Rat No.	Diet.	Body weight.	Food eaten in 24 hrs.	Weight of liver.	Liver glycogen.		Body glycogen.		Intestinal content, reducing sugar.	Blood sugar, weight per 100 cc.	Total glycogen.	
					Weight.	Per cent of liver.	Weight.	Per cent of body weight.			Weight.	Per cent of body weight.
		gm.	gm.	gm.	mg.		mg.		mg.	mg.	mg.	
51	Control.	335	22	16	205	1.28			97			
5	"	258	13	12	77	0.64			66			
1	"	224	16	12	97	0.81			517			
2842	"	205	8	10	92	0.93			106			
4	"	146	16	8	74	0.93			181			
3	"	154	12	9	156	1.70			62			
106	"	114	12	7	91	1.30	79	0.069	39		170	0.149
107	"	150	12	7	92	1.31	101	0.067	36		193	0.128
108	"	152	15	7	108	1.54	129	0.084	64		237	0.156
112	"	148	12	7	91	1.30	80	0.054	39		171	0.115
109	"	138	12	7	68	1.40	134	0.097	35		202	0.146
Average.	Control.		14		106	1.18 ± 0.069	108	0.074		173 ± 6		0.137
Standard deviation.....			0.32				28		
110	Fasted.	202	0	7			13	0.006		125		
111	"	150	0	6			13	0.009		129		
2901	"	156	0	7			57	0.036	8	111		
2868	"	130	0	5	43	0.08	11	0.009	16	127		
Average.	Fasted.							0.015		123		

31	Almond.	343	13	14	51	0.36	28	0.020	10	114	53	0.038
41	"	328	26	16	64	0.40			5			
101	"	140	10	7.8	25	0.31	25	0.018	6	111	51	0.036
102	"	142	10	7.6	26	0.34	21	0.013	5			
103	"	156	8	7.4	30	0.40	25	0.012	3	128	51	0.032
104	"	196	8	8.4	30	0.35	57	0.025	3	127	55	0.028
105	"	224	11	8.9	29	0.32	37	0.020	4	141	86	0.038
2840	"	188	8	8.0	41	0.52	60	0.045	19	148	78	0.041
2845	"	133	12	6.0	36	0.60	65	0.046	13		96	0.072
2832	"	137	14	8.0	57	0.72			30		122	0.089
Average.	Almond.		12		39	0.43 ± 0.046		0.026		128 ± 4		0.047
Standard deviation.	0.13				14		

Lohmann (36) found that when muscle glycogen is split, it yields lactic acid immediately, because of the presence of glycolytic ferments; thus in fasting animals, the muscle glycogen might yield lactic acid which on reaching the liver might be deposited as glycogen. Cori and Cori (37) found this increase in liver glycogen at the expense of muscle glycogen following injections of epinephrine. In the light of these findings, the disappearance of body glycogen and retention of liver glycogen in our control rats may be explained as due to the somewhat prolonged period of fasting preceding the day or two before the glycogen-forming test was made. The same consideration need not apply to the almond-fed rats, however, since these animals ate the almond meal greedily from the beginning. Yet both body and liver glycogen were found to be depleted almost to the fasting level. The actual quantity of liver glycogen found in the control rats was nearly 3 times that found in the almond-fed rats, and this is also the proportion of available sugar shown previously to be present in the almond carbohydrate as compared with the completely assimilable corn-starch of the control diet.

These findings indicate the possible usefulness of almonds or almond meal in low carbohydrate diets such as are used for diabetics and in the ketogenic dietetic treatment of epileptic children. The bulky fecal residue resulting from the use of the nut, which is probably partly due to the unabsorbable carbohydrate, indicates the laxative character of this food.

SUMMARY.

1. Almonds of different crops and varieties were found to vary in proximate composition and in the carbohydrate fraction particularly.

2. A defatted almond meal of approximately 30 per cent "carbohydrate by difference" content was submitted to analysis in order to determine the nature of the carbohydrates present, with the following results: (a) Sucrose made up nearly one-third, 27.6 per cent, of the carbohydrate, and 2.3 per cent of the blanched almonds used, which had 8.5 per cent total carbohydrates. (b) Pentosans accounted for 24.7 per cent of the carbohydrate or 2.1 per cent of the almonds. (c) Crude fiber was 13.7 per cent of the carbohydrate or 1.2 per cent of the almonds. (d) Total

acid-hydrolyzable carbohydrate was 47.8 per cent of the "carbohydrate by difference" and undetermined substances in the carbohydrate fraction represented 41.6 per cent of that fraction.

3. The digestibility of the almond carbohydrate was tested by feeding to rats a diet made up of the almond meal and almond oil and also a control diet of the usual purified type with corn-starch replacing the almond carbohydrate. The weights of both fresh and dried feces excreted by the almond-fed rats were 2 to 3 times as great as those excreted by the control rats. Pentosans and crude fiber found in the almond feces represented only 8.5 per cent and 27 per cent respectively of those substances in the food eaten. Bacterial decomposition may account for this loss. The marked laxative effect of the almond meal is noted.

4. Rats fed the same almond and control diets were fasted for 24 hours, fed the diets for 24 hours, then killed, and blood sugar, liver glycogen, and body glycogen determined. In all cases the almond-fed rats showed lower blood sugar values and total glycogen contents than the control rats. The proportion of glycogen in the almond-fed rats was about one-third that in the control rats, a condition which corresponds closely with the proportion of available carbohydrate obtained in the chemical studies reported above.

5. The value of almonds and almond meal for use in both constipation and diabetes, and also in making up an acceptable ketogenic diet for epileptic children is suggested by these results.

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THE INFLUENCE OF CEREALS UPON THE RETENTION OF CALCIUM AND PHOSPHORUS IN CHILDREN AND ADULTS.*

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The present research was undertaken to determine first the retention of calcium and phosphorus by children and adults on a diet fairly high in cereals and second the difference, if any, in the retention of these minerals when refined wheat products and oatmeal products formed a part of the diets. Mellanby's startling results in the development of rickets in dogs by the use of varying amounts of cereals and the fact that cereals form a large part of the diet of human beings make the study of these foods important.

HISTORICAL.

Mineral metabolism experiments with cereals are surprisingly limited in number and are concerned chiefly with animals. In some experimental studies on rickets in puppies, Mellanby (1, 2) found that on a rachitic diet containing white bread or various types of cereals, the severity of the condition increased with the amount of cereal product eaten and that oatmeal caused the worst rickets while white bread was the least rickets-producing of all of the cereals. Experimentation with the fat of the oatmeal and whole wheat showed that the saponifiable fraction was responsible for the "antagonistic" effect, existing to a greater degree in oatmeal than in wheat germ.

Green and Mellanby (3) in a repetition of this experiment, using rats instead of dogs, obtained similar results and noted that various supplements

* The experimental data in this paper are taken from a thesis submitted by Helen Brown Burton in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Ogden Graduate School of Science in the University of Chicago, 1929.

such as cod liver oil, or hydrolysis of the starch in the cereal overcame the rachitic effect.

Steenbock (4) in a brief report of similar experimental work on both rats and dogs stated that in rats the oats were slightly better than maize and less desirable than wheat, but that in larger animals such as dogs all of the cereals were so rickets-producing that many carefully controlled investigations carried out over a long period of time are necessary before one may decide which cereal is least satisfactory so far as rickets is concerned.

Another study of cereals was that by Cowgill, *et al.* (5) in which wheat, corn, and oat cereals were fed at 63 to 93 per cent levels. With such supplements as meat residue, liver, salt mixture, cod liver oil, lard, and lettuce, or in another experiment (6) egg, molasses, and lettuce, he obtained excellent results with rats; oats and wheat could be used at a higher level than corn without producing rickets or faulty dentition.

A somewhat similar investigation by Rose and MacLeod (7) in which white bread plus various supplements as dried milk, dried egg, dried vegetables, or meat were used, showed that even with as much as 30 per cent dried milk or egg results were not very satisfactory, for it was difficult to rear the young even to weaning time and those that did live were in poor condition, frequently definitely rachitic.

Practically all of the rachitic diets used in experimental work contain some type of cereal in rather large amounts. Also, one knows from various studies such as the one by Pearl (8) and from personal observations of the eating habits of people that cereals form an important part of the diet of the human race.

In the present study the effect of two types of cereal products, rolled oats and refined wheat, upon calcium and phosphorus retention in human subjects, especially children, was investigated with the hope of adding to the information concerning the mineral values of these foods.

EXPERIMENTAL.

Subjects.—Human subjects were used, during the first experiment two girls, 12 and 13 years of age, and two adults, and in the second experiment four boys ranging in age from 3 to 5 years. All of the subjects were in fairly good physical condition, except possibly the two girls. All were weighed at the beginning and end of the experiment. They lived away from home during this time in an apartment with the author, and the children were under constant supervision.

General Procedure.—The first experiment, that with the two girls and the two adults, lasted from July 29 to August 14, 1926,

and the second, in which the four boys were the subjects, from January 18 to February 4, 1927. The experimental period was 5 to 6 days on each diet with a 3 day preliminary period and 1 day lag.

During the first experiment as the weather was sunshiny and pleasant, the girls were outdoors considerably. There was approximately the same amount of sunshine during both periods. The boys played outdoors even though the weather was unpleasant and cloudy. As these younger children were from a nursery school where radiations by a mercury vapor quartz lamp had been recently added to the care given the children,

TABLE I.
Distribution of Irradiation.

Subject.	Preliminary period.	Oat period.	Lag period.	Preliminary period.	Wheat period	Lag period.
H. H.	No irradiation.	Total, 3 min., 4th and 5th days.	No irradiation.	No irradiation.	Total, 18 min., 2nd to 5th days.	No irradiation.
Other three boys.	Total, 6 min., 3 days.	Total, 15 min., 1st, 4th, and 5th days.	No irradiation.	No irradiation.	Total, 33 min., 2nd to 5th days.	No irradiation.

these plans were not changed. Since the radiations may have affected the retention of the minerals by the boys, the number of minutes of radiation received by the children and the distribution between periods will be given in detail. The radiations at first lasted 1 minute daily and by the end of the experimental period 9 minutes daily. 7 days out of the entire time, as the nursery school was closed, the boys did not receive any radiations. For three of the boys the total time during the oat period was 21 minutes and during the wheat period 33, while for H. H., as he was not irradiated until the 4th day of the experimental oat period, the time was 3 minutes during the oat period and 18 during the wheat period. Table I shows the distribution between the two periods.

Diet.—The plan was to use approximately average amounts of cereals in the diets, one type of cereal only being used during each period instead of varying the cereal as is the custom in normal diets. No doubt the amount of cereal is higher than is eaten ordinarily by people able to afford adequate diets, but it is probably less than the amount used by poorer people. With individuals accustomed to a varied diet it is rather difficult to incorporate large amounts of cereals such as are used in animal experiments.

The basal diet during the first experiment with the girls and adults consisted of canned peaches, ground lean beef, riced potatoes, canned peas, lettuce, filtered butter fat, sugar, and dried milk. To these foods were added Cream of Wheat or steamed rolled oats, white bread or oatmeal muffins, and a pudding made of white bread or muffins, sugar, cocoa, and a minimum amount of eggs and milk. In the second experiment, that with the boys, the diet differed in that the oats were fed the first period, oatmeal crackers were used in place of muffins, cookies made of the appropriate cereal instead of the pudding, canned tomatoes in place of peas, the potatoes were omitted, and the cereal was fed at both the morning and evening meals. Steamed rolled oats were used in both experiments except for some steel cut oatmeal in the crackers in the diet of the four boys. Distilled water was used for drinking and for mixing with the dried milk.

The diets for the adults in Experiment I were the same as for the two girls except that the amount of milk was reduced in order to bring the calcium intake down to the minimum on which equilibrium could be maintained, and Adult 2 ate less meat and potato than the other subjects.

Computing the acid-base residue of the diets from Sherman and Gettler's figures (9), one finds that in both of these experiments the wheat diet is slightly basic and the oat diet approximately neutral. The difference is too insignificant to have any marked effect upon the retention of the minerals during the two periods.

Collection of Excreta.—The urine and feces for each subject for each period were kept separate, the urine being preserved with hydrochloric acid and toluene and the feces treated with ethyl alcohol and hydrochloric acid, dried to constant weight, pulver-

ized, and stored in air-tight bottles. Carmine was used to mark off the stools.

Food Samples.—Samples of all foods except butter fat and sugar were kept for analysis. The dried milk, cereals, oatmeal cakes, and the cookies were stored without further drying, the peaches and tomatoes were canned, and the other foods dried to constant weight previous to storing. The calcium in meat and the phosphorus in lettuce, because of the low content of the foods in these minerals, were calculated from Sherman's figures (10).

Analysis.—McCrudden's method (11) was used for all calcium determinations, the pH for the precipitation of calcium oxalate in food and feces being adjusted to fall between 4.8 and 5.2 (12). Food and feces were dry-ashed and prepared for analysis according to the official method (13). The total phosphorus in the urine was determined by the official method (13). Food and feces were carefully wet-ashed with nitric and sulfuric acids and the analyses made. In all of the phosphorus determinations the precautions outlined by McCandless and Burton (14) were observed.

DISCUSSION OF RESULTS.

Physical Condition of the Children.—The children were in good health the entire time of the study. While the girls gained only $\frac{1}{2}$ to $\frac{3}{4}$ pound each, the boys each gained $\frac{3}{4}$ to $1\frac{1}{4}$ pounds.

Calcium and Phosphorus Intake.—In Table II are the figures for the composition of the foods used in the experiments and for comparison figures taken from Sherman's tables (10). The daily intakes of two of the individuals for the two experiments, typical of all of the subjects, are shown in Tables III and IV. The highest percentage of calcium came from the milk, but the cereal products were the next most important source. In both individuals the cereals furnished more phosphorus than calcium, especially during the oat period.

The amount of mineral in the diet was higher for the younger children than for the two girls, especially for calcium (Tables V and VI). For neither group was the intake the same on both diets, being higher in calcium on the wheat diet and higher in phosphorus on the oat diet, with the exception of N. R. for whom both of the minerals were higher on the oat diet (Tables V and VI). With the girls the attempt was made to adjust the calcium intake

on the oat diet as it was expected that the whole cereal would increase the amount, with the result that the intake was lower than during the wheat period. Even in the boys' diets in which no change was made except the cereal, the calcium intake was lower on the oat diet.

TABLE II.
Composition of Foods in Percentages.

Food.	Calcium.					Phosphorus.				
	Experiment I.		Experiment II.		Sherman.	Experiment I.		Experiment II.		Sherman.
	Period 1.	Period 2.	Period 1.	Period 2.		Period 1.	Period 2.	Period 1.	Period 2.	
Cream of										
Wheat	0.021			0.022	0.021	0.110			0.110	0.125
Oatmeal		0.049	0.054		0.069		0.342	0.342		0.392
Bread	0.060			0.068	0.027	0.096			0.103	0.093
Muffins		0.049					0.191			
Oatmeal										
cookies			0.116					0.317		
Vanilla										
cookies				0.160					0.244	
Oat cakes			0.117					0.470		
Pudding	0.133	0.108				0.128	0.123			
Canned										
peaches	0.004	0.006	0.005	0.007	0.016	0.015	0.014	0.013	0.013	0.024
Canned peas .	0.023	0.020			0.026	0.034	0.037			0.127
" tomatos										
toes			0.009	0.012	0.011			0.020	0.021	0.026
Meat, lean										
beef, cooked.	0.014	0.014	0.014	0.014		0.155	0.144	0.205	0.189	
Potato, "	0.008	0.007				0.015	0.014			
Lettuce	0.013	0.009	0.011	0.011	0.043	0.015	0.014	0.014	0.014	0.042
Milk, dried. .	0.995	1.012	0.943	1.015	0.960*	0.733	0.754	0.750	0.737	0.744*

* This is 8 times the value given for whole milk, inasmuch as dried milk diluted to 8 times its weight is equivalent to cow's whole milk.

In Tables V and VI are the per kilo intakes of Sherman and Hawley's subjects (15) and one notes that the intake in both of the minerals by the boys was $1\frac{1}{2}$ to 3 times as much as Sherman and Hawley's children, while for one girl it was approximately the same and for the second girl lower than the other figures.

In the case of the boys the intake figures show that on a diet containing generous amounts of cereals it is possible to have a rather high mineral intake, even with refined wheat products.

Calcium Balance.—The two girls were in positive calcium balance on the wheat diet, but on the oat diet, while L. S. retained

TABLE III.

Distribution of Calcium and Phosphorus in Diet of One Girl, L. S.

Food.	Daily intake.	Ca	P		
Period 1.					
	gm.	gm.	per cent of total	gm.	per cent of total
Cream of Wheat	28	0.006	1	0.031	3
Bread.....	126	0.083	8	0.121	10
Pudding.....	118	0.157	16	0.151	13
Canned peaches	220	0.009	1	0.033	3
" peas.....	116	0.026	3	0.088	7
Potatoes.....	171	0.012	1	0.026	2
Lettuce.....	70	0.009	1	0.011	1
Meat.	174	0.023	2	0.267	22
Milk, dried.....	65	0.645	66	0.475	40
Total.		0.970		1.203	
Period 2.					
	gm.	gm.	per cent of total	gm.	per cent of total
Oatmeal.....	25	0.013	2	0.046	3
Muffins.....	270	0.133	20	0.515	38
Pudding.....	120	0.127	19	0.148	11
Canned peaches	220	0.013	2	0.031	2
" peas.....	117	0.023	3	0.087	6
Potatoes.....	171	0.012	2	0.024	2
Lettuce.....	70	0.006	1	0.009	1
Meat.	174	0.023	3	0.250	2
Milk, dried.....	32	0.324	48	0.241	18
Total.....		0.674		1.351	

a slight amount of calcium, M. S. lost some (Table V). Storage per kilo of body weight on the wheat diet compared favorably with Sherman and Hawley's subjects (15) of the same age, but not on the oat diet.

The four little boys retained calcium during the two dietary

periods, but both the actual amounts stored and the percentages were higher on the wheat than on the oat diet (Table V). The younger children on either diet retained much larger amounts and percentages than the girls, even on the oat diet storing an average of 46 per cent while the most the girls stored was an average of

TABLE IV.

Distribution of Calcium and Phosphorus in Diet of One Boy, R. E.

Food.	Daily intake.	Ca	P		
Period 1.					
	gm.	gm.	per cent of total	gm.	per cent of total
Oatmeal.....	75	0 040	3	0 258	14
Oat cakes..	56	0 065	5	0 264	15
“ cookies.....	44.7	0 052	3	0 162	9
Canned peaches. .	200	0 010	1	0 025	1
“ tomatoes..	115	0 010	1	0 023	1
Lettuce.....	10	0 001		0 001	
Meat.....	40	0 004		0 082	5
Milk, dried.	128	1.207	87	0 960	54
Total.		1.389		1 775	
Period 2.					
	gm.	gm.	per cent of total	gm.	per cent of total
Cream of Wheat	56	0.014	1	0 061	4
Bread.....	112	0 076	5	0 115	8
Vanilla cookies....	60	0.128	8	0 195	14
Canned peaches.	200	0 013	1	0 025	2
“ tomatoes.	115	0 013	1	0 022	2
Lettuce... ..	10	0.001		0 001	
Meat.....	40	0 004		0.076	5
Milk, dried.....	128	1.299	84	0 943	66
Total.....		1 548		1 438	

29 per cent. The retentions per kilo were slightly better during the wheat than during the oat period, and on the oat or wheat diet were considerably higher than those of the girls, Sherman and Hawley's children (15) or Willard and Blunt's subjects (16), being 4 to 5 times as large as the average for Sherman and Hawley's children of the same age (Table V). In a forthcoming book by Blunt and Cowan (17) in which retentions by children are tabu-

lated, the figures for the boys in the present experiment were much higher than those reported by any other investigators.

TABLE V.
Calcium Metabolism.

[illegible]

tions were likewise less on the oat diet, but the decrease in retention was much greater than the decrease in intake as may be seen in Table VII. Except in L. S. the retention was decreased about 0.10 gm. per person per day more than the intake was decreased. Even N. R. who had a slight increase in intake over the wheat period intake retained less. All of the boys and one of the girls excreted more calcium on the oat diet than on the wheat.

Phosphorus Balance.—The differences in the retention of phosphorus on the two diets were more striking for the girls than for the boys, for while the girls stored one-fourth of the intake on the wheat diet, on the oat there was loss (Table VI). The retentions per kilo of body weight on the wheat diet were as satisfactory as those found by other investigators (15).

In the four little boys the variations in retention of phosphorus during the two periods were not clear cut, for two of the boys retained less of this mineral on the oat diet and two slightly more. The percentage retentions of phosphorus on the wheat diet were better for all of the younger children except H. H. (Table VI). As in the case of calcium storage the phosphorus retentions per kilo of body weight were much higher than figures reported by other investigators (15, 17) (Table VI). Possibly these higher retentions of both minerals by the four boys were due to the rather high mineral intake, to the calcium : phosphorus intake ratios, and to the ultra-violet radiations received by the younger children.

Although the phosphorus intake during the oat period was increased over what it was during the wheat period, the retentions were decreased in four of the children while in the other two only one-third to one-half of the added phosphorus was retained (Table VII).

Channels of Excretion.—In all except one case the fecal calcium loss was greater during the oat than during the wheat period (Table V). The differences were more striking in the case of phosphorus, for the losses through the feces were increased 2 to 5 times what they were when the subjects were on the wheat diet (Table VI). The percentages of fecal calcium and phosphorus were likewise increased on the oat diet, especially so in the case of the phosphorus. Other investigators (18) have noted that in rachitic infants the fecal phosphorus was much higher than in

cases of healing rickets or in normal infants. The percentages of urinary excretions of both minerals were decreased on the oat diet and in almost all cases the actual amounts were reduced, but not enough to make up for the added fecal loss. Sjollema (19) investigating the effect of crude fiber upon mineral retention found that increasing the fiber increased the fecal calcium, but only slightly affected the phosphorus output. As in the present ex-

TABLE VII.
Differences in Intake and Retention of Calcium and Phosphorus.

Subject.	Age.	Differences in intake between oat and wheat periods.		Differences in retention between oat and wheat periods.	
		Ca	P	Ca	P
	<i>yrs.</i>				
L. S., girl.	12	-0 29	0 14	-0.21	-0.33
M. S., "	13	-0 29	0.14	-0 43	-0.52
H. H., boy.	5	-0 17	0 30	-0 21	0 15
N. R., "	3	0 05	0 33	-0 06	0.11
R. E., "	3	-0.12	0.25	-0 22	-0 05
L. D., "	3	-0 19	0 24	-0 28	-0.15

TABLE VIII.
Average Daily Weights of Dry Feces.

Subject.....	L. S.	M. S.	Adult 1.	Adult 2.	H. H.	N. R.	R. E.	L. D.
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Wheat.....	21	16	18	22	14	17	18	14
Oats.....	30	32	23	36	21	24	26	25

periment the urinary calcium was decreased, but by an amount insufficient to make up for the fecal loss.

In this connection it is interesting to note that in every case the dry weights of the feces when the subjects were on the oat diet were greater than they were on the wheat diet, from 27 to 100 per cent greater (Table VIII). Shohl and Bennett (20) noted that when dogs became rachitic the weights of the feces increased.

Calcium : Phosphorus Ratios.—As the calcium intake was lower and the phosphorus intake higher on the oat than on the wheat diet, the calcium : phosphorus intake ratios were lower on the oat

than on the wheat diet (Table IX). The difference was considerable even in the four boys in whose diets the only change made was the cereal. The calcium:phosphorus retention ratios were

TABLE IX.
Calcium:Phosphorus Ratio.

Subject.	Intake.		Retention.	
	Wheat.	Oats.	Wheat.	Oats.
L. S.	0.8	0.5	0.9	
M. S.	0.8	0.5	1.0	
H. H.	1.1	0.6	1.3	0.8
N. R.	0.9	0.6	1.1	0.9
R. E.	1.1	0.8	1.3	1.1
L. D.	1.1	0.8	1.4	1.2

TABLE X.
Mineral Metabolism in Adults.

Subject.	In- take.	Urine.	Feces.	Total out- put.	Reten- tion.		
Calcium metabolism.							
	gm.	gm.	per cent of ex- cretion	gm.	per cent of ex- cretion	gm.	gm.
Adult 1.							
Wheat.	0.65	0.25	36	0.46	64	0.71	-0.06
Oats.	0.51	0.17	29	0.43	71	0.59	-0.08
Adult 2.							
Wheat.	0.63	0.08	11	0.61	89	0.68	-0.06
Oats	0.50	0.08	9	0.79	91	0.88	-0.38
Phosphorus metabolism.							
Adult 1.							
Wheat.	0.98	0.79	83	0.16	17	0.95	0.03
Oats.	1.23	0.80	65	0.59	35	1.39	-0.16
Adult 2.							
Wheat.	0.83	0.57	69	0.26	31	0.82	0.01
Oats	1.10	0.83	29	0.86	71	1.69	-0.59

also lower on the oat than on the wheat diet, being negative in the case of the two girls, no doubt partly due to the low calcium:phosphorus intake ratio. In including considerable oat products

in the diet, the calcium intake should be kept rather high so that the calcium:phosphorus intake ratio will be more satisfactory. The retention ratios of all of the subjects while on the wheat diet compared fairly well with some of Daniels' subjects, especially in the case of the four boys, but they did not at any time reach the high ratio, 2, found by Daniels (18) in infants receiving irradiated milk. The fact that the calcium:phosphorus retention ratios in the little boys were higher than in the girls might have been due to the larger intake of minerals by the younger children, to the more satisfactory calcium:phosphorus intake ratios, or to the radiations received by the boys, probably a combination of all three conditions.

Mineral Metabolism in the Adults.—The mineral intake for the adults was lower than that for the children. Both individuals were in negative calcium balance during both periods, but the loss was greater on the oat than on the wheat diet, only slightly so for Adult 1, but considerably greater for Adult 2 (Table X). While phosphorus was stored on the wheat diet, on the oat diet, even with an increase in intake, there was greater excretion. The percentage of fecal calcium and phosphorus was always higher on the oat than on the wheat diet, especially so in the case of fecal phosphorus. The actual amounts of fecal phosphorus and except in Adult 1 the amounts of fecal calcium were likewise larger on the oat than on the wheat diet. The reduction in urinary excretion was insufficient to make up for the added fecal loss. As in the children the dried feces during the oat period weighed more than during the wheat period.

SUMMARY.

A metabolism experiment was conducted to determine the retention of calcium and phosphorus by six children and two adults on cereal diets and the differences in retention when oat products were used and when wheat products were used.

The retentions of calcium and phosphorus by four boys on either cereal diet were exceedingly high, possibly due to the rather high intakes and possibly to the radiations received by the boys. The girls did not retain as much of the intake as the boys did.

The retentions were higher on the wheat than on the oat diet.

More mineral was excreted through the feces and the weights of the dried feces were greater on the oat than on the wheat diet.

In general the results for the adults were similar to those for the children; namely, less retention on the oat diet, and greater fecal loss and greater weight of dried feces on the oat than on the wheat diet.

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AN INQUIRY INTO THE EXISTENCE OF INTERMEDIATE COMPOUNDS IN THE OXYGENATION OF HEMOGLOBIN.

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The recent determinations of the molecular weight of hemoglobin (1, 2) have established the fact that there are 4 atoms of iron per molecule. The problem, therefore, arises as to whether or not stable compounds exist in which 1 or more of the iron atoms are chemically different from the others. For example, in the oxidation of hemoglobin (ferrous compound) to methemoglobin (ferric compound) (3-5) are all 4 iron atoms oxidized at once or does the reaction proceed in definite steps corresponding to stable compounds containing 1, 2, and 3 ferric atoms respectively? If one assumes that there are no intermediate compounds, then the oxidation-reduction potential of the hemoglobin-methemoglobin system should correspond to Equation 1 in which $n = 4$. The experimental results presented in a paper published 2 years ago

$$(1) \quad E = E'_0 - \frac{RT}{nF} \ln \frac{\text{Hb}}{\text{MHb}}$$

(5) showed that the actual value of n is about 2. A similar anomaly exists in regard to the combination of oxygen with hemoglobin. On the basis of the new molecular weight and no intermediates the exponent n in the usual oxygen dissociation equation should be 4; $\frac{Z}{1-Z} = K P^n$ where Z is the degree of oxygen saturation and P the pressure of the oxygen. Actually it is nearer 2 though it varies with the degree of oxygenation and the pH of the solution. We suggested a few years ago (6) a possible explanation of the peculiarities of the oxygen dissociation curve in connection with our work on the adsorption of nitrogen by the protein. We

are now convinced that this explanation is fundamentally unsound although the question of the adsorption of oxygen by hemoglobin is still unsettled. Furthermore, no explanation connected with the behavior of gases will solve the difficulties raised by the unexpected value of n in the electrochemical equation of the hemoglobin-methemoglobin system.

The most hopeful way out of the difficulties which confront one in formulating either the oxidation or oxygenation of hemoglobin is to assume the existence of intermediate compounds. Adair (7) first showed that if certain additional assumptions are made in regard to the free energy of each successive step in the process, it is possible to arrive at an equation which closely corresponds to the facts. Ferry and Green (8) have used this procedure in the formulation of their extensive measurements of the equilibrium between oxygen and hemoglobin in buffer solutions of varying acidity. Their equations are most successful in reproducing the experimental results. The reversible oxidation reactions involving hemoglobin and methemoglobin may be treated satisfactorily in exactly the same way,¹ though unfortunately the experimental data are not sufficiently accurate to provide a test comparable to that furnished for the oxygenation reaction by Ferry and Green's measurements.

Thus, it appeared that if the existence of stable intermediates in the oxygenation or oxidation reactions could be experimentally established many of the difficulties connected with the quantitative study of hemoglobin would disappear. We therefore sought to obtain experimental evidence for the existence of intermediates in the oxygenation of hemoglobin. We have now to report that

¹ It is interesting to note that although the oxidation and the oxygenation of hemoglobin are analogous reactions, custom more or less dictates the form of the equations and the graphical methods employed. Thus if Z is the fraction of oxygenated or oxidized hemoglobin and P the pressure of oxygen or a measure of the escaping tendency of electrons ($E = \frac{RT}{nF} \log P$) the relation of Z and P may be plotted in the following ways: (a) Z against P , the usual dissociation curve of oxyhemoglobin, *not* used in electrochemistry; (b) Z against $\log P$, the usual electrochemical method *not* used in connection with dissociation curves; (c) $\log \frac{Z}{1-Z}$ against $\log P$ (or $-\log P$), used in testing the Hill equation, and occasionally in electrochemistry.

the results of our study point strongly to the opposite conclusion, and we are convinced that some other explanation of the anomalies must be sought.

I. Composition of Solid Oxyhemoglobin.

Oxyhemoglobin corresponding to the formula Hb_4O_8 (where Hb equals the unit with 1 iron atom) is a crystalline compound of definite solubility (Cohn and Prentiss (9)); hemoglobin (Hb_4) is much more soluble. The possible intermediates Hb_4O_6 , Hb_4O_4 , and Hb_4O_2 would therefore be expected to be more soluble than the completely oxygenated compound. Nevertheless, it is possible that one or more of them might separate as a solid phase from a solution which contained very little combined oxygen and therefore a large amount of the intermediates,—if they, indeed, exist. We have examined the composition of the solid phase in equilibrium with a solution of hemoglobin in a phosphate buffer of pH = 6.60 when the solution was saturated with oxygen to the extent of only 7.9 per cent of its capacity. As will be shown in detail later, the amount of intermediates in such a solution should be very large according to Ferry and Green's equations. Since the concentration of actual solid phase in the paste obtained by centrifugation is only about 15 to 20 per cent, the analysis is necessarily complicated and not very accurate.

EXPERIMENTAL.

Preparation of Equilibrium Mixture.—Horse hemoglobin was used throughout this work. It was purified as crystalline oxyhemoglobin according to the method of Ferry and Green (8) except that the laking of the cells was accomplished by freezing and thawing. The crystalline oxyhemoglobin was washed to constant solubility at 0° in the desired buffer by the procedure recommended by Cohn and Prentiss (9). Using a phosphate buffer of pH 6.60 and ionic strength 2.20, we found a solubility of 3.40 gm. per 100 cc. The centrifuged paste of oxyhemoglobin crystals that remained after washing to constant solubility was placed in a 1 liter tonometer together with 10 or 20 per cent of its weight of the same buffer solution. The tonometer was then evacuated, shaken for about 5 minutes, and filled with nitrogen, all the operations being performed in a room at 2°. The process was repeated until the

gradually increasing purple color indicated that enough oxygen had been pumped out to leave a considerable amount of deoxygenated hemoglobin. The tonometer containing the mixture was then slowly rolled for several hours, or even a few days, in a bath of ice and water at 0° in order to establish an equilibrium between the solid, liquid, and gaseous phases.

Sampling.—In order to obtain a sample for analysis, a portion of the mixture in the equilibration tonometer was drawn, in nitrogen, into a smaller evacuated tonometer that could be centrifuged, the latter consisting merely of a 250 cc. centrifuge bottle fitted with a rubber stopper carrying a 3-way stop-cock. In order to avoid loss or gain of oxygen during the centrifugation, as soon as the sample had been introduced the remaining space in the bottle was filled with gas from the large equilibration tonometer, and finally enough nitrogen run in to make the pressure slightly more than atmospheric. After centrifuging the mixture hard enough so that the layer of solid that settled out would not move when the bottle was inverted, the solution was drawn out of the inverted bottle and filtered in the absence of oxygen. The apparatus for this purpose consisted of a fluted filter paper in a Gooch funnel closed at the top with a rubber stopper carrying a 3-way stop-cock, and connected at the bottom to another tonometer. The air in this apparatus had previously been replaced by nitrogen and the nitrogen then replaced by gas drawn from the large equilibration tonometer, the final pressure being left a little less than atmospheric. To avoid leaks all rubber stoppers were sealed with shellac. As soon as the solution had been drawn from the centrifuge bottle into the filter paper in the Gooch funnel, more nitrogen was run into the latter to give a pressure slightly more than atmospheric. Then, as the filtrate came through it was drawn into the tonometer below, from which samples for analysis could finally be taken conveniently. Usually about 30 minutes were required for the centrifugation and 15 for the filtration, so the first sample could be analyzed an hour after it had been drawn from the equilibration tonometer, all of the operations of centrifugation, filtering, etc., during that time having been conducted in a room at about 2°.

Analysis of Solution.—The amount of oxygen in the filtrate was determined and also the additional amount that it would absorb

on being shaken with air. The amounts of methemoglobin and cathemoglobin² were also determined in some cases. The measurement of the amount of oxygen originally present in the filtrate was made by simply transferring a 2 cc. sample, at 0°, in nitrogen, to the Van Slyke manometric apparatus, where the oxygen was liberated and measured in the usual way. It was assumed that all of the oxygen in the unsaturated solution was "bound oxygen." Another sample of the filtrate was then shaken with air at room temperature for about 10 minutes, during which time enough oxyhemoglobin often crystallized to render the mixture too viscous to measure readily with a pipette, in which case a small amount of ammonia vapor was blown into the mixture, with stirring, until the crystals redissolved. A 2 cc. sample of the oxygenated solution was then analyzed gasometrically as before, a correction of 0.50 volume per cent being allowed for dissolved oxygen. The amount of oxygen unsaturation was given by the difference between the amounts of oxygen found in these two analyses. To determine the amount of methemoglobin present another sample of the filtrate, usually before shaking with air, was reduced with titanous tartrate, then shaken with air, and analyzed for oxygen gasometrically, according to the method of Conant, Scott, and Douglass (10). The amount of methemoglobin present was given by the difference between the amounts of bound oxygen found in this analysis and in the preceding one where the solution was shaken with air without preliminary reduction. Finally, cathemoglobin was determined by carbon monoxide capacity, another sample of the filtrate being reduced with ammoniacal sodium hydrosulfite solution and then saturated with carbon monoxide (11). To avoid a large solubility correction the saturating gas consisted of 2 per cent carbon monoxide in nitrogen, which gives a carbon monoxide pressure sufficient to saturate completely both the hemoglobin and hemochromogen present in the slightly alkaline solution with a solubility correction of only 0.04 volume per cent of CO. The carbon monoxide was determined with the modified manometric apparatus and procedure of Harington and Van Slyke

² We shall use the term cathemoglobin (CHb) in this paper to designate any denatured form of hemoglobin that is insoluble at the neutral point but soluble in either weak acid or alkali and which after reduction has a carbon monoxide capacity but no oxygen capacity.

(12). The amount of cathemoglobin present was given by the difference between the amount of bound carbon monoxide thus obtained and the amount of bound oxygen found in the preceding methemoglobin analysis.

In order to be certain that the equilibrium mixture still contained an excess of crystalline oxyhemoglobin a 2 cc. sample of it was transferred, in nitrogen, directly to the Van Slyke apparatus and its oxygen content determined for comparison with that of a sample that had been centrifuged and filtered.

Analysis of Solid Phase.—The weighed paste remaining in the centrifuge bottle was dissolved in distilled water and a little ammonia, both free from oxygen. By having the solution slightly alkaline with ammonia and keeping it at 0° it was found that practically all of the oxygen remained in the solution, although if the temperature was allowed to rise an appreciable amount of oxygen passed from the solution to the nitrogen above it in the bottle. By gasometric analyses of aliquot portions the amounts of oxyhemoglobin, reduced hemoglobin, methemoglobin, and cathemoglobin were determined in just the same way as had been done with the filtrate. From the results of these analyses of the solution the amounts of the various forms of hemoglobin in the centrifuged paste could be calculated by knowing the weight of that paste, the weight of the solution of it, and the weight of the samples analyzed.

Calculation of Composition of Solid.—In all cases it was found that the amount of oxygen that could be taken up by a 2 cc. sample of the centrifuged paste of solid was considerably less than that taken up by 2 cc. of the corresponding filtrate, thus suggesting that the unsaturation in the paste was due entirely to the solution that it still contained. This conclusion was verified by determining the amount of solution that actually remained in the paste. An approximate knowledge of this was furnished by the gasometric analyses themselves, for, if the carbon monoxide capacity showed the paste to contain a total of, say, 25 per cent protein, it could be estimated, from the analysis of the filtrate and its density, how much of the total protein was present as actual solid phase and how much was dissolved in the solution which the paste still contained, and how much completely deoxygenated

hemoglobin was present in the solution. The calculation is as follows:

Let A = gm. of total protein in 1 gm. of filtrate (given by total oxygen capacity of filtrate and its density)

B = gm. of total protein in 1 gm. of centrifuged paste (given by its CO capacity)

y = gm. of actual solid phase in 1 gm. of centrifuged paste

z = gm. of solution in 1 gm. of centrifuged paste

Then $y = 1 - z$ and $B = y + zA = 1 - z + zA$, whence $z = \frac{B - 1}{A - 1}$

Then, if C = amount of deoxygenated hemoglobin in 1 gm. of the filtrate (given by the oxygen unsaturation and density), zC = amount of deoxygenated hemoglobin (oxygen unsaturation) in the solution in 1 gm. of the centrifuged paste, which value can be compared with that found by actual analysis.

An independent check was made in some cases by drying a sample of the centrifuged paste to constant weight at about 120°. The loss in weight showed the amount of water that it had contained and one could then estimate, from the analysis of the filtrate, how much reduced hemoglobin must have been present in that water.

The data and the results of these calculations for two typical samples are given in Table I. The two columns represent measurements made by approaching the equilibrium from both sides. Those in the first correspond to the experiment carried out as just described. The data in the second column were obtained with the same solution after it had been completely deoxygenated and oxygen again admitted; thus, the oxyhemoglobin in the second case had crystallized from a solution which had been free from this compound. The deoxygenation of the solution was accomplished at 2° by pumping and filling the tonometer with nitrogen; the solution was centrifuged to remove any cathemoglobin, before oxygen was again added to the gaseous phase in contact with it.

In Part A of Table I are given the experimental data. The figures in Section 3 were calculated from those in Section 1 and the density of the filtrate (1.132); the composition of the paste (Section 6) was directly on a weight basis. The final figures in Section 6 give the experimentally determined oxygen unsaturation of the paste expressed as gm. of completely deoxy-

TABLE I.

Composition of Centrifuged Paste and Solid Phase.

Phosphate buffer, pH = 6.60, ionic strength = 2.20, equilibrium at 0°.

	Equilibrium approached from:	
	Oxygenated side.	Deoxygen- ated side
A. Experimental data.		
1. Concentration of oxyhemoglobin in filtrate (gm per 100 cc. of solution).	1.24	1.66
After saturation with air.	15.8	14.3
2. Density of filtrate	1.132	1.132
3. Concentration of oxyhemoglobin in filtrate (gm. per gm. of solution).		
(a) Direct.	0.0110	0.0147
(b) After saturation with air	0.1396	0.1263
(c) Deoxygenated hemoglobin, difference.	0.1286	0.1116
4. Per cent by weight of potassium phosphate in buffer solution.	15.1	15.1
5. Per cent by weight of H ₂ O in centrifuged paste (from dry weight)	58.1	62.1
6. Concentration of total hemoglobin in centrifuged paste (gm. per gm. of paste).		
(a) Total protein, by CO.	0.2650	0.2553
(b) Oxyhemoglobin, direct.	0.1425	0.1038
(c) " after saturation with air.	0.2218	0.1754
(d) Deoxygenated hemoglobin, difference.	0.0793	0.0716
B. Calculation of unsaturation in paste. Method 1.		
Fraction of solution in centrifuged paste (z) (calculated from Part A, Sections 3 (b) and 6 (a)).	0.854	0.853
Fraction of solid phase in centrifuged paste (y).	0.146	0.147
Calculated unsaturation due to solution in paste (gm. Hb per gm. paste).	0.110	0.095
C. Calculation of unsaturation in paste. Method 2.		
Gm. potassium phosphate in 1 gm. centrifuged paste . .	0.1034	0.1105
" buffer solution " 1 " " "	0.6844	0.7315
" deoxygenated Hb in solution in 1 gm. centrifuged paste	0.101	0.092

generated hemoglobin per gm. of paste. These figures are to be compared with the amount of deoxygenated hemoglobin in the paste by virtue of its presence in the liquid phase. This amount is calculated by two methods, as explained above. The calculations are given in Parts B and C of Table I. Part B follows the equations given above; in Part C the per cent of water in the paste determined by drying (Part A, Section 5) and the known composition of the buffer (Section 4) and of the filtrate (Section 3) enable one to calculate directly the amount of deoxygenated hemoglobin in solution in 1 gm. of the paste (last figure, Part C).

TABLE II.

Summary of Data Obtained in Examination of Paste.

The results are expressed in gm. per 100 gm.

Equilibrium approached from:	Amount of deoxygenated hemoglobin in paste				
	Calculated in liquid phase.		Average calculated.	Total found (Section 6 (d), Table I).	Difference.*
	Method 1.	Method 2			
Oxygenated side.	11.0	10.1	10.5	7.9	-2.6
Deoxygenated "	9.5	9.2	9.3	7.2	-2.1

* The difference between the found and calculated (last column) would be as follows for different compositions of solid phase (since the total solid phase in 100 gm. of paste is about 16 gm.): Hb_4O_8 , 0; Hb_4O_6 , +4.0; Hb_4O_4 , +8.0; Hb_4O_2 , +12.0.

A check on the two methods is given by calculating the total protein in 1 gm. of the paste from (a) the dry weight and (b) the CO analysis (Van Slyke procedure). In the first experiment the figures were 0.316 and 0.265 respectively and in the second 0.269 and 0.255, as good an agreement as could be hoped for.

DISCUSSION.

The final results of our examination of the solid phase in the two cases given in detail in Table I are summarized in Table II. The amount of deoxygenated hemoglobin found in the paste is actually somewhat less than the amount which our calculations indicate should be present in the liquid phase. If the solid phase contained less oxygen than Hb_4O_8 , the difference would be in the other direc-

tion by the amounts noted in Table II. Thus, in spite of the admitted uncertainties of ascertaining the nature of the solid phase in a 16 per cent paste, we feel reasonably sure that the solid phase is Hb_4O_8 and no evidence for the existence of intermediates can, therefore, be found in these experiments.

II. Existence of Intermediates More Soluble Than Oxyhemoglobin.

Since the solid phase in equilibrium with the solution appears to be the well known oxyhemoglobin (Hb_4O_8), the question of the existence of soluble intermediates can be tested by the analysis of the solution provided the solubility of Hb_4O_8 is independent of the presence of reduced hemoglobin or possible intermediates. Landsteiner and Heidelberger's experiments (13) showed that the oxyhemoglobins of different species have solubilities which are practically independent of each other. It thus seems probable that the solubility of Hb_4O_8 would be nearly independent of the presence of other molecular species of hemoglobin. The amount of Hb_4O_8 in a solution saturated with respect to this solid phase should be the same whether or not the solution was wholly Hb_4O_8 (completely oxygenated) or contained considerable amounts of Hb_4 or the intermediates (largely deoxygenated). However, the intermediates (Hb_4O_6 , Hb_4O_4 , Hb_4O_2), if they exist, also carry oxygen and the total amount of combined oxygen in any solution will be the sum of this amount and that supplied by the oxyhemoglobin (Hb_4O_8). If the intermediate compounds existed it is clear that there should be more combined oxygen in a saturated solution of oxyhemoglobin, which was also unsaturated with respect to its oxygen capacity, than in the same solution saturated with oxygen and therefore containing no intermediates. Indeed, the equations of Ferry and Green (8) enable one to calculate this increase of combined oxygen which is to be predicted from the theory of intermediates. The total concentration of combined oxygen is given by Equation 2, the last term of which corresponds to the amount of oxygen carried by the oxyhemoglobin (Hb_4O_8):

(2) Concentration of total O_2 =

$$[\text{Hb}_4]_T' (K'_1 [\text{O}_2] + 2K'_1 K'_2 [\text{O}_2]^2 + 3K'_1 K'_2 K'_3 [\text{O}_2]^3 + 4K'_1 K'_2 K'_3 K'_4 [\text{O}_2]^4)$$

Dividing Equation 2 by this term we obtain the ratio of the concentration of combined oxygen to the amount present as Hb_4O_8 , Equation 3.

(3) Ratio of total combined O_2 to O_2 as Hb_4O_8 =

$$\frac{K'_1 [\text{O}_2] + 2K'_1 K'_2 [\text{O}_2]^2 + 3K'_1 K'_2 K'_3 [\text{O}_2]^3 + 4K'_1 K'_2 K'_3 K'_4 [\text{O}_2]^4}{4K'_1 K'_2 K'_3 K'_4 [\text{O}_2]^4}$$

As an example of the application of this equation we may substitute the values of K given by Ferry and Green for $\text{pH} = 6.55$ and 25° , and an oxygen pressure of 7 mm. corresponding to a saturation of 8 per cent ((8), Fig. 2) which leads to Equation 4.

$$(4) \quad \text{Ratio} = \frac{0.4493}{0.0804} = 5.59$$

The solubility of Hb_4O_8 in the solution when it is saturated with oxygen is given by the oxygen content of this solution (corrected for physical solubility of oxygen). When such a solution is brought to a condition of 92 per cent unsaturation with respect to oxygen, while the concentration of Hb_4O_8 is kept constant, the combined oxygen should increase 5.6-fold. This predicted effect is so enormous that there should be no difficulty in detecting it, if intermediates existed in any such amounts as would be necessary to account for the shape of the oxygen dissociation curve.

In order to obtain sufficiently low solubilities to test the effect, it is necessary to work at 0° instead of 25° . However, there seems to be no reason to assume that this change of temperature would greatly alter the *shape* of the oxygen dissociation curve. In other words, like the change of pH , a slight alteration of temperature would be expected to affect the individual values of K_1 , K_2 , etc., but not their relation to each other. In practice, the calculated increase in the oxygen content of the solution would be limited by the amount of total hemoglobin available, which in our experiments was about 25 gm. per 100 cc. of the equilibrium mixture. Thus, if the initial solubility of Hb_4O_8 was 1 gm. per 100 cc. it follows from Equation 3 that when the total hemoglobin concentration became 25 gm. per 100 cc. (*i.e.*, as the last of the Hb_4O_8 crystals dissolved) the total combined oxygen would be 3.18 and the solution would be 12.7 per cent saturated with oxygen. Simi-

TABLE III.

Oxygen Content of Solutions Saturated with Oxyhemoglobin and Containing Large Amounts of Deoxygenated Hemoglobin.
 pH = 6.60, ionic strength = 2.20, temperature = 0°, initial solubility = 3.40.

Total No. of evacuations.	Time for equilibration.	Sample No.	Day of mo. taken.	Material analyzed.	Composition of sample (gm. oxyhemoglobin per 100 cc. solution).							Change in HbO ₂ solubility.	Satura- tion.	
					Direct.	After saturation with air.	Differ- ence = Hb.	MHb		CHb				gm. per 100 cc.
								O ₂ capac- ity after reduc- tion.	Differ- ence = MHb.	CO capac- ity after reduc- tion.	Differ- ence = CHb.			
105	12	1	6	Filtrate.	1 24	15.8	14.56	15.0	0					
				Solid.	14 25*	22.18	7.93	22.8	0.6	26.5	3.7			
				Filtrate.	0 86									
155	19	2	7	Equilibrium mixture.	8 8									
228	0	3	8	"	3.7									
245	21	5	12	Entire mixture centrifuged; ppt. (48 gm.) discarded; 125 cc. air added to solution (337 gm.).										
				Filtrate.	1.66	14 3	12 64	13.8	0					
				Solid.	10.38	17.54	7 16	19 8	2.3	25 5	5.7			
22	6	13	Filtrate.	1.71										
50 cc. more of buffer solution added; tonometer filled with air.	21	7	15	Filtrate.		3.55								

* Figures for the composition of the centrifuged paste are expressed as gm. per 100 gm. of paste; all other figures in gm. per 100 cc.

larly, if the solubility of Hb_4O_8 were 4 gm. per 100 cc., the oxygen concentration should be 6.8 and the solution 27 per cent saturated when the last of the solid phase dissolved.

The data obtained in one experiment are given in full in Table III. The experiment corresponding to Table III is the one for which the analysis of the solid phase (Tables I and II) was discussed. A pH of about 6.60 was taken as corresponding to the

TABLE IV.

Summary of Analyses of Liquid Phase Saturated with Oxyhemoglobin.

Experiment No.	pH	Ionic strength.	Solubility of Hb_4O_8 in oxygenated solution.	Analysis of partially deoxygenated solutions at equilibrium (gm per 100 cc.).				Per cent saturated with O_2 .	Change in oxygen content.	
				Hb	Hb_4O_8	MHb	CHb		Found (0°).	Calculated from Ferry and Green, pH = 6.55, 25° .
									per cent	per cent
1	6.60	2.20	3.40	14.56	1.24	0		7.9	-63.5	+505
1a*	6.60	2.20	3.40	12.64	1.66	0		11.6	-51.2	+255
2*	6.60	0.49	5.40	12.81	1.53	4.55	2.6	10.7	-71.7	+290
3	6.42	3.0	0.94	6.23	0.80			11.4	-14.9	+265
4	6.42	3.0	0.94	0.79	0.78	0		49.7	-17.0	+35
4a	6.42	3.0	0.94	8.13	1.12			12.1	+19.1	+240
5	6.54	0.25	6.24	19.29	2.91			13.1	-53.3	+210

* Equilibrium was approached from the side of completely deoxygenated solution. In Experiment 1a the mixture was finally completely oxygenated and equilibrium established. The concentration of Hb_4O_8 in solution was then found to be 3.55 as compared with 3.40 at the beginning of the experiment.

minimum solubility of oxyhemoglobin (9). Relatively concentrated buffer solutions were employed so that very little if any change in pH occurred as more protein dissolved during the deoxygenation. In almost every case two or more analyses of the solution were made, the tonometer being rotated for at least several hours between analyses. These analyses were consistent and showed that equilibrium had been established. The concordant results obtained by approaching the equilibrium from both sides

were very gratifying as was also the fact that after a complete cycle the solubility of Hb_4O_8 in an oxygenated solution was unchanged.

It is clear from the data presented in Table IV that instead of the large increase in oxygen content and the early passage of all the oxyhemoglobin into solution which we expected to find, we actually observed in most cases a decrease in oxygen concentration and a persistence of solid Hb_4O_8 when the solutions had reached degrees of deoxygenation far beyond those at which the theory would predict its disappearance. The observed oxygen decrease is large but not of the order of magnitude of the increase predicted on the basis of the existence of intermediates. We are inclined to attribute this diminished solubility of oxyhemoglobin in solutions rich in deoxygenated hemoglobin to something of the nature of a "salting out effect" of the one protein or the other. It is possible that such an effect might mask the increase in oxygen content which we were endeavoring to find, and it might explain, qualitatively, the persistence of solid oxyhemoglobin in equilibrium with highly deoxygenated solutions. But it is evident from an inspection of the last two columns of Table IV that one would have to postulate an enormous effect of this sort, indeed. Aside from this, there seems no escape from the conclusion that if there are intermediates in the oxygenation of hemoglobin they are present in too small amounts to account for the anomalies in the oxygen dissociation curve. We are personally convinced that some other explanation of the peculiarities of hemoglobin must be sought.

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ON THE ORIGIN OF INOSINIC ACID.

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The chemical relationship between the simple purines, adenine and hypoxanthine, has long been known, the latter being the deaminization product of the former. The same relationship might be expected, on theoretical grounds, between adenine nucleotide and hypoxanthine nucleotide (inosinic acid). Inosinic acid, first isolated by Liebig (1), has been looked upon as a primary constituent of muscle. The secondary origin of inosinic acid from the deaminization of adenine nucleotide was not suspected because pentose adenine nucleotide was not known to occur in animal tissues.

The isolation (2) of adenine nucleotide from pig blood and the demonstration (3) of its occurrence in comparatively large quantities in the blood of several species suggested to the author the possibility that adenine nucleotide may be the precursor of inosinic acid in muscle, and may be decomposed during contraction to yield inosinic acid and ammonia. If true, this reaction would have bearing upon several problems of metabolism such as the origin of endogenous purines in urine, the effect of exercise on certain phases of nitrogen metabolism, and of the intermediary metabolism of phosphoric acid and carbohydrate, and the acid-base relationships during the cycle of muscular contraction and relaxation.

If muscles are indeed capable of deaminizing adenine nucleotide in the body with the formation of inosinic acid and ammonia, aqueous extracts of muscle under appropriate conditions should deaminize *added* adenine nucleotide. Equivalent amounts of

* A part of this work, during the summer of 1928, was done at the University of Wisconsin through the courtesy of Professor E. B. Hart and Professor H. Steenbock.

inosinic acid should appear in the digests, over and above that found in the control extracts. Or, what is easier of quantitative demonstration, the "extra" purine should be demonstrable after hydrolysis of the nucleotide as hypoxanthine if deamination has occurred; as adenine, if deamination has not occurred. There remains the theoretical possibility that phosphoric acid may have been split off from the adenine nucleotide and that the adenosine so formed may have been subsequently deaminized. In this event the inorganic phosphate would have been increased. This proved not to be the case. In no case was the inorganic phosphate of the digests appreciably increased.

In this paper are reported results obtained with muscles of several species by the method just outlined. In brief it was found that the voluntary muscles of the pig and ox regularly and completely deaminized added adenine nucleotide prepared from yeast nucleic acid; muscles of the rabbit and human being deaminized yeast adenine nucleotide although in certain instances not completely; and rat muscles under the same conditions did not deaminate yeast adenine nucleotide appreciably.

Recently Embden (4-8) and coworkers, Schmidt (9), and Parnas (10) have reported extensive work undertaken from a different view-point and using different methods, which bears upon the same problem. Embden (4) isolated from rabbit muscle a compound which he first regarded as identical with yeast adenine nucleotide. Later he found that the yeast and muscle adenine nucleotides were not identical, as evidenced by certain physical and chemical differences. Also he made the important observation that ammonia is given off when frog muscle is stimulated (5) and when muscle adenine nucleotide is added to extracts of frog or of rabbit muscle; but that it is not given off when yeast adenine nucleotide is added. Consequently inosinic acid may be produced by muscle from *muscle* adenine nucleotide but not from *yeast* adenine nucleotide.

Inosinic acid is a nucleotide which on hydrolysis yields the purine hypoxanthine, the pentose *D*-ribose, and orthophosphoric acid, the pentose being the central or connecting group. A sharp distinction has been drawn between nucleic acids (and nucleotides) of plant and of animal origin based upon the nature of the carbohydrate group, the plant products having a pentose

and the animal products an unknown carbohydrate commonly regarded in the past as a hexose, and now regarded by Levene (11) as a desoxypentose. This distinction is inconsistent in view of the finding of all the known pentose nucleotides which occur primarily in yeast nucleic acid in animal tissues (12).

Heretofore there has been no reason for suspecting a chemical difference between pentose nucleotides containing the same base. Jones and Perkins (13) found the adenine and cytosine nucleotides prepared from the β -nucleoprotein of the pancreas identical with those prepared from yeast, and Hoffman (2) found the adenine nucleotide prepared from blood identical with that prepared from yeast. Embden's (14) discovery of a second pentose adenine nucleotide in the animal organism is based upon differences in the behavior of yeast and muscle adenine nucleotides in respect to their melting point, specific rotation (of the sodium salt), solubility, rate of hydrolysis of the phosphoric acid, and the apparent pentose content as determined by the Hoffman method.

In 1918 Jones and Kennedy (15) prepared crystalline adenine nucleotide from yeast nucleic acid and analyzed the compound. The chemical properties of adenine nucleotide do not lend themselves to its easy identification. Since adenine nucleotide does not melt, "melting points" are meaningless. The solubility of adenine nucleotide in water is interesting because of its tendency toward the formation of highly supersaturated solutions, which, when crystallization takes place, yield a product which is surprisingly insoluble in water. Such a product may be recrystallized twice from hot water if sufficiently large volumes are used, but the decreasing solubility of the product obtained renders further recrystallization futile.

It is well known (16) that phosphoric acid is much more readily split by acid hydrolysis from the purine nucleotides than from the pyrimidine nucleotides. Yamagawa (17) observed that inosinic acid resembles the pyrimidine nucleotides in this respect, rather than the purine nucleotides. Levene and Jacobs (18) oxidized the phosphoribose prepared from inosinic acid and obtained phosphoribonic acid and concluded that in inosinic acid the phosphoric acid is attached to the terminal carbon atom. Levene and Mori (19) adduced more evidence to uphold this view. Accepting this allocation of the phosphoric acid in inosinic acid, Yamagawa

(17) concluded that the allocation of the phosphoric acid must be the same in the pyrimidine nucleotides but different in the purine nucleotides. This argument is not convincing in the light of the experiment of Levene and Jorpes (20) who found that the simple hydrogenation of cytosine nucleotide (which could not have influenced the allocation of the phosphoric acid) so far altered the rate of hydrolysis of the phosphoric acid as to make it resemble purine rather than pyrimidine nucleotides in this respect. Obviously in this case the nature of the base rather than the allocation of the phosphoric acid is the predominating factor in determining the rate of hydrolysis of the phosphoric acid. Levene and Jorpes (20) believe that the phosphoric acid of both pyrimidine and purine nucleotides is attached to a secondary carbon atom.

Whatever may be the allocation of the phosphoric acid in these compounds there is now ample evidence of two types of pentose adenine nucleotide in nature; the yeast and the muscle type. One must expect corresponding hypoxanthine nucleotides, etc. In the light of these new developments the terms *adenine nucleotide* or *adenylic acid*, *hypoxanthine nucleotide* or *inosinic acid* are not specific. At present the rate of hydrolysis of the phosphoric acid seems to be the best chemical criterion of distinction between muscle and yeast adenine nucleotides. Hoffman did not report the "partial phosphorus" (that phosphorus which is split from the compound by hydrolysis for $2\frac{1}{2}$ hours with 7 per cent sulfuric acid in a boiling water bath) for his blood adenine nucleotide, a determination which might be expected to distinguish between the yeast and muscle compounds. If the blood compound proves actually to be of the yeast type the muscles must either convert the yeast type into the muscle type of adenine nucleotide or synthesize the muscle type *de novo*. The results in this paper indicate that the muscles of several species are provided with the mechanism necessary to deaminize either directly or indirectly the yeast type of adenine nucleotide and also the muscle type.

EXPERIMENTAL.

The classical method of studying enzyme action was followed; that is, a known quantity of a chemically pure compound was allowed to react under suitable conditions with an extract of the tissue under investigation, and certain products of the reaction were isolated quantitatively and identified.

Muscle was dissected from the animal as soon as possible after death and put through a meat grinder. It was extracted with twice its weight of water, to which a little chloroform was added, for 1 hour on a shaking machine. The extract was pressed through cheese-cloth, and the residue was reextracted in the same way. Aliquot portions of the combined extracts were used, one portion serving as a control. 100 gm. or more of muscle were represented in each aliquot. A solution of the compound in water, or dilute alkali when appropriate, was added (an equal volume of water was added to the control), the solution was brought to the desired pH, 10 cc. of chloroform were added, the flask was loosely stoppered, and allowed to incubate at 37° for the length of time designated, the mixtures being shaken several times each day.

The digested mixtures were treated as follows. The reaction was adjusted to pH 6, the solution was brought rapidly to a boil to coagulate the proteins, and was filtered. Enough sulfuric acid was added to make the filtrate 7 per cent H_2SO_4 and the solution was kept just below the boiling point for 1 hour. Concentrated sodium hydroxide was added until the solution reacted only *faintly* acid toward litmus. Purines were then precipitated as the cuprous compounds by means of copper sulfate and sodium bisulfite at the boiling temperature. The copper compounds were decomposed with hydrogen sulfide, the excess of which was boiled out. The solution was made alkaline to litmus with ammonia, and ammoniacal silver nitrate was added as long as a precipitate continued to form. This precipitate was collected, and washed with water until free from ammonia. It was suspended in hot water and decomposed with an excess of hydrochloric acid. The silver chloride was filtered off, and the filtrate was evaporated to dryness over a water bath, care being taken to avoid superheating. The residue was taken up twice with small quantities of water, and twice with alcohol, followed by careful evaporation to expel free hydrochloric acid. Finally the residue was dissolved in water, and a saturated aqueous solution of picric acid was added. If adenine was present a voluminous precipitate of adenine picrate formed immediately; it was filtered off after an hour or two, dried, and weighed. If adenine was not present no precipitate formed immediately and only an insignificant quantity after several hours.

TABLE I.
Deamination of Adenine Nucleotide by Muscle Extracts.

Species.	Experiment No.	Compound added.	pH	Time.	Nature of purine recovered.								Remarks.
					Adenine picrate.				Hypoxanthine nitrate.				
					Control.	Total.	Extra purine.	per cent	Control.	Total.	Extra purine.	per cent	
Ox.	1 a	Blank.	6.0	11	0	201	201	95	66	182	116	97	Control. Neck muscles. Extract boiled. Complete deamination. Almost complete deamination.
	b	200 mg. yeast adenine nucleotide.	6.0	11		0	201	95	64	170	104	87	
	c	"	6.0	11		0	0						
	d	"	7.4	11		30	30	14					
	e	"	9.0	11		81	81	40					
Fig.	1 a	Blank.	6.0	10	0	521	521	95	137	111	45	38	Partial deamination. Control. Leg muscles. Extract boiled. Complete deamination. Control. Mixed muscles of four rats.
	b	500 mg. yeast adenine nucleotide.	6.0	10		0	521	95		413	242	93	
	c	"	6.0	10		0	0						
Rat.	1 a	Blank.	6.0	4	0								Complete deamination. Control. Mixed muscles of four rats. Probably no deamination.
	b	200 mg. yeast adenine nucleotide.	6.0	4		160	160	76					
Rabbit.	1 a	Blank.	6.0	5	0				193				Control. Mixed voluntary muscles. Complete deamination.
	b	200 mg. yeast adenine nucleotide.	6.0	5		0	0			304	111	93	

Rabbit.	2 a	Blank.	6.0	2	0		133	133	63	141					Control. Mixed voluntary muscles.
	b	200 mg. yeast adenine nucleotide.	6.0	2			133	133	63		183	42	35		Only partial deamination.
	c	200 mg. muscle adenine nucleotide.	6.0	2			0	0			248	107	90		Complete deamination.
Human.	1 a	Blank.	6.5	10	0					20					Control. Died of a nervous affection.
	b	200 mg. yeast adenine nucleotide.	6.5	10			160	160	76		37	17	14		Insignificant deamination.
Human.	2 a	Blank.	6.0	1½	0					43					Control. Acute peritonitis, pneumonia, etc.
	b	200 mg. yeast adenine nucleotide.	6.0	1½			0	0			147	104	87		Complete deamination.
	c	200 mg. muscle adenine nucleotide.	6.0	1½			0	0			153	110	92		" "

These solutions, whether adenine had been found or not, were further investigated for hypoxanthine as follows. After dilution with several volumes of water the purines were once more precipitated as cuprous compounds, which were decomposed with hydrogen sulfide. The copper sulfide was filtered off, the filtrate was evaporated to dryness on the water bath, and the residue was crystallized out of 5 per cent nitric acid. After standing several hours the hypoxanthine nitrate was filtered off, dried, and weighed.

DISCUSSION.

Results reported in Table I demonstrate that muscles of certain species can deaminate adenine nucleotide both of yeast and of muscle origin. Hypoxanthine nucleotide is usually found in muscles. When extreme precautionary measures are taken to prevent enzymatic deamination, adenine nucleotide is found.

Hypoxanthine nucleotide, then, must be expected in all ordinary muscle extracts, but no adenine nucleotide; hypoxanthine in these extracts after hydrolysis, but no adenine. This proved to be the case (Table I). Muscle extracts preserved with chloroform retain their activity as regards the deamination of adenine nucleotide for several days. If, however, a fresh extract is boiled, it no longer has any deaminizing power (Table I). In such cases 95 per cent of the purine present in the added adenine nucleotide was recovered in the hydrolyzed solution as adenine picrate, and in addition the expected quantity of hypoxanthine nitrate due to the presence of hypoxanthine nucleotide in the muscle extract (Table I, ox muscle, Experiment 1). If, however, the muscle extract was not boiled, no adenine whatever was found and recovery of the purine present in the added adenine nucleotide was satisfactory (about 90 per cent of the theoretical) as hypoxanthine nitrate, over and above the quantity shown by the control (blank) to have been originally present in the extract. These facts not only demonstrate the thermolabile nature of the deamination, but also serve as a check on the experimental method. In view of the large numbers of procedures involved in each experiment and the small quantities of material used, these recoveries were considered entirely satisfactory.

The power of organs and tissues of different species to catalyze chemical changes in compounds derived from yeast nucleic acid

has been extensively studied by Jones (21) and others. Nowhere is specificity of enzyme action more vividly illustrated. The results in Table I also illustrate this point. For instance rat muscle failed to deaminate yeast adenine nucleotide, in contrast to the other muscles studied. Too much stress cannot be laid upon this point, however, because of paucity of data at the present time, but attention is called to the danger of carrying over results obtained with one species to another.

Another point, again illustrated by data in Table I, is the fact that reactions can sometimes take place when a compound is conjugated with a second grouping, which cannot proceed on the simple compound. For example no human tissue can deaminate free adenine (22), whereas human muscle can deaminate both adenosine and adenine nucleotide (Table I). That adenine nucleotide is not deaminized through the intermediate formation of adenosine is indicated by the failure of inorganic phosphate to increase during incubation.

Leonard and Jones (23) pointed out the fact that hypoxanthine is always found in muscle tissue in spite of the fact that this tissue is peculiarly poor in nucleic acid and that the voluntary muscles of certain species (pig, dog, and rabbit) contain no adenase. The hypoxanthine which could not be formed directly by the deaminizing action of adenase they called "preformed hypoxanthine." The origin of preformed hypoxanthine was in doubt. The explanation is now clear: Pig muscle (see Table I) deaminizes adenine nucleotide. The hypoxanthine nucleotide thus formed must be the precursor of the hypoxanthine found.

No attempt was made to determine exactly the optimum pH for the deamination of adenine nucleotide. It was found, however, that faintly acid media (pH 6) were suitable for this reaction, whereas at pH 7.4 the reaction proceeded more slowly (Table I). Distinctly alkaline media (pH 9), which Kay (24) found to be optimum for the action of the phosphatases of mammalian tissue on guanine nucleotide, proved to be unsatisfactory for the deamination of yeast adenine nucleotide. Embden found a sharp optimum pH at 5.8 for the deamination of muscle adenine nucleotide.

The second experiment with rabbit muscle is particularly noteworthy because it illustrates the simultaneous action of the

same muscle extract on yeast adenine nucleotide and on muscle adenine nucleotide. The muscle adenine nucleotide was prepared by the author's method (to be published shortly) from beef hearts. The yeast adenine nucleotide was prepared in the usual way from Merck's yeast nucleic acid. At the end of 2 days more than half of the yeast adenine nucleotide remained unchanged whereas all of the muscle adenine nucleotide had been deaminized. Provisionally this experiment may be interpreted as indicating a greater ease of deaminization of the muscle than of the yeast adenine nucleotide. Possibly Embden's failure to find that ammonia is given off when rabbit muscle is stimulated is due to this fact. That rabbit muscle can deaminize yeast adenine nucleotide is shown in Experiment 1.

Human muscle was obtained as soon as possible after death, and cannot be regarded as strictly normal. In the first experiment only 14 per cent of the yeast adenine nucleotide was deaminized. More significant is the second experiment in which all of the yeast adenine nucleotide was deaminized, as was also the muscle adenine nucleotide. In spite of the unusually short period of incubation this experiment failed to distinguish between the yeast and the muscle type of compound. The fact seems established that human muscle can deaminize yeast adenine nucleotide.

SUMMARY.

1. The voluntary muscles of the ox, pig, rabbit, and human being have the power of deaminizing adenine nucleotide prepared from yeast nucleic acid, with the formation of hypoxanthine nucleotide. Under the same conditions of experimentation rat muscles did not deaminize yeast adenine nucleotide appreciably.

2. This deaminization takes place rapidly in faintly acid media (pH 6), more slowly at 7.4, and very slowly at 9.

3. The deaminization of adenine nucleotide by muscle explains the regular occurrence of inosinic acid in muscle tissue.

4. It also explains the old riddle of the occurrence of the hypoxanthine found in certain muscles which contain no adenase.

5. Muscle adenine nucleotide proved in certain cases to be more readily deaminized than yeast adenine nucleotide.

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STUDIES ON METABOLISM IN PNEUMONIA.

I. THE EXCRETION OF "ORGANIC ACID" AND A METHOD FOR ITS DETERMINATION.

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The concept of an increased formation and excretion of acids in pneumonia seems to have first found experimental justification in the work of Palmer and Henderson (1), who found that the administration of 4 gm. of sodium bicarbonate did not appreciably change the reaction of the urine of a patient with pneumonia. In normal individuals this amount of bicarbonate produced a change in reaction of approximately pH 1.7. Later, Palmer (2) determined the buffer values of urine from a pH of 7.4 (neutral red) to one of pH 5.0 (alizarin). After subtracting the buffer value of the phosphate content, it was found that the remainder was, in some pneumonia urines, considerably greater than in normal urine. This increase was ascribed to the presence of organic acids.

The technique for the determination of organic acid was greatly improved by Van Slyke and Palmer (3). Phosphate was precipitated by means of $\text{Ca}(\text{OH})_2$, thus obviating the necessity for a phosphate determination and a correction based thereon. Van Slyke and Palmer recognized that weak bases would titrate as do weak acids by their technique and they introduced corrections for the creatinine and creatine content of the urine. They mentioned the possibility of the presence of other bases of unknown nature but considered this to be extremely unlikely.

Using this method, Clausen (4) made a number of determinations of the organic acid content of the urine of children with pneumonia. He found that large quantities were excreted and that the maximum output coincided with the period of resolution.

Holten (5), on the other hand, reported that the excretion of organic acid was diminished after crisis. Both Clausen and Holten agreed that ether-soluble acids played little or no part in the increased excretion of organic acid.

Neither Clausen, Holten, nor Palmer corrected their values for the creatinine and creatine content of the urine, nor do they seem to have given sufficient consideration to the fact that protein catabolism is usually greatly increased in pneumonia and that if the organic acid was derived from protein, the amount excreted would be correspondingly increased without any qualitative change in metabolism.

The subject seemed worthy of further investigation. 24 hour specimens of urine were obtained from a large number of patients with pneumonia in Harlem Hospital. Most of these patients were so ill that they were being kept in the oxygen, or atmosphere control, room. In all, 102 specimens were obtained. In these, organic acid was determined by the method of Van Slyke and Palmer. Only four showed a gross content (uncorrected for creatinine and creatine) of more than 1000 cc. of 0.1 N acid. These and two additional urine samples from the same patients but with slightly lower organic acid content, and one other urine sample were removed to this laboratory for further study. Here, the titration of organic acid was repeated. The end-point of the titration was found to be difficult of determination because of the intense color of the urine. Neither of the indicators, tropeolin OO and brom-phenol blue, recommended by Van Slyke and Palmer, gave really satisfactory results. However, there could be no question but that the amount of "organic acid" in these urine samples, even after correction for their creatinine and creatine content, was greater than in normal urine. The ratio of "organic acid" to total nitrogen was also higher than in normal urine.

With the intention of obtaining a colorless liquid in which to perform the titration and also in order to obviate the necessity for creatinine and creatine determinations, portions of these urine samples were treated with Lloyd's reagent, as in the method of Folin and Berglund (6) for the determination of sugar. The acid filtrates were treated with calcium hydroxide and the titration was then completed in the usual manner, correction being made for the titration obtained in a control determination. It was

found that there was now no such difference between the ratio of "organic acid" to nitrogen in pneumonia urine and the same ratio in normal urine. In other words, the treatment with Lloyd's reagent had removed a substance, or substances, of unknown nature, that had marked buffer qualities between pH 8.0 and 2.7. Suitable experiments showed that formic, acetic, lactic, and β -hydroxybutyric acids added to urine could be nearly quantitatively recovered in the filtrates from Lloyd's reagent.

Other samples of these urines were made acid to Congo red with sulfuric acid. Phosphotungstic acid was then added until further addition no longer produced a precipitate. Aliquots of the filtrate were treated with powdered, anhydrous barium hydroxide until slightly alkaline and were then filtered. Titration of these filtrates gave values for "organic acid" that were intermediate between those obtained with the use of Lloyd's reagent and those obtained without such treatment. The amount of buffer substance precipitated by phosphotungstic acid was far greater in the pneumonia urine samples than it was in the normal urine. That the unknown substance was precipitated by the phosphotungstic acid and not by the barium hydroxide was shown by a control titration.

Other portions of urine were treated with copper sulfate and calcium hydroxide. Titration of the filtrates showed that only a small part of the buffer substances had been precipitated.

These observations would seem to indicate that the unknown substance is not an acid but a base or an ampholyte. If so, it would probably contain nitrogen. Analyses of the urine samples for ammonia, urea, creatinine, creatine, and uric acid account for all but from 4.0 to 7.5 per cent of the total nitrogen. This is in accord with the results obtained by Wolf and Lambert (7).

The maximum excretion of the unknown substance observed in these experiments seems to have occurred in the case of Subject M on March 19. The difference between the values obtained by the method of Van Slyke and Palmer, corrected for creatinine and creatine, and those obtained by the titration of the filtrates from Lloyd's reagent was the equivalent of 1207 cc. of 0.1 N acid. This was accompanied by an excretion of nitrogen that was twice as great as in one normal subject (Subject G) and nearly 4 times as great as in another (Subject L). On the assumption that the

increase in nitrogen metabolism was accompanied by a correspondingly large increase in the amount of this buffer substance excreted, the urine should have contained the equivalent of at least 325 cc. of 0.1 N acid from this source. Not more than 882 cc. of the difference in the amounts found by the two methods can be ascribed to a qualitative change in metabolism due to the pneumonia. On the assumption that the unknown substance binds 1 equivalent of acid for each atom of nitrogen in the molecule, this amount of acid is equivalent to 1.235 gm. of nitrogen, or 4.2 per cent of the total nitrogen in the urine. Subtraction of this figure from that for the undetermined nitrogen, 6.8 per cent, leaves 2.6 per cent. This value is within the range observed by Folin (8) in normal individuals with a daily nitrogen excretion of not more than 0.3 gm. of nitrogen per kilo of body weight. In a dog fed only meat and excreting 1.5 gm. of nitrogen per kilo per day, Schöndorff (9) found that 95 per cent of the total nitrogen was present as urea. Other constituents were not determined but the "undetermined nitrogen," in the usual sense, could not have amounted to more than about 1 per cent. It is, therefore, quite likely that the "organic acid" excreted by some patients with pneumonia is, in reality, a nitrogenous base or ampholyte.

The nature of the substance will be further investigated as soon as additional material is secured.

SUMMARY.

The titration of organic acid in urine is greatly facilitated by previous treatment of the urine with Lloyd's reagent. This makes correction for creatinine and creatine unnecessary¹ and gives a colorless filtrate in which, after treatment with $\text{Ca}(\text{OH})_2$ to remove phosphates, the titration is readily performed. The blank is greater than when $\text{Ca}(\text{OH})_2$, alone, is used. Although added formic, acetic, lactic, and β -hydroxybutyric acids are practically completely recovered, the values obtained are considerably lower than those obtained by the method of Van Slyke and Palmer. The method would seem to be suitable for clinical use in diabetes.

Previous reports concerning a high organic acid content in the

¹ Creatine is not completely removed but the amount left in the filtrate is negligible.

urine of patients with pneumonia may have been erroneous because the values obtained were not corrected for the creatine and creatinine content of the urine and because proper consideration was not given to the effect of the increased metabolism. As determined in the filtrate from a Lloyd's reagent precipitation, the organic acid content of the urine is not out of proportion to the nitrogen content. However, in certain pneumonia urine samples, there is a considerable quantity of a substance of unknown nature, which has buffer properties between pH 8.0 and 2.7 and which is removed by treatment with Lloyd's reagent or phosphotungstic acid but not by treatment with calcium hydroxide, with or without the addition of copper sulfate. The amount of this substance, or substances, may be far greater than can be accounted for by the increased protein catabolism.

The cases studied were from the Pneumonia Service, Harlem Hospital, in charge of Dr. J. G. M. Bullowa, to whom I am indebted for his interest and cooperation. I am also indebted to Mr. Marvin Lowenthal, who made the Van Slyke-Palmer determinations at the Harlem Hospital station of this fund and to Mr. Irving Levy, who did most of the rest of the experimental work.

EXPERIMENTAL.

All determinations were made in duplicate. In one titration, tropeolin OO was used as indicator; in the other, brom-phenol blue. The results generally agreed very well. The values reported represent averages.

Correction for Creatine.—Contrary to the experience of Van Slyke and Palmer, who reported that creatine titrated to about 60 per cent of the value calculated for a mono-acid base, we have found that it titrated to only about 43 per cent of that amount.

Amount taken.	Calculated.	Found.	Per cent of calculated.	Indicator.
gm.	cc. 0.1 N	cc. 0.1 N		
0.203	15.5	6.74	43.4	Tropeolin OO.
0.201	15.3	6.85	44.7	Brom-phenol blue.
0.200	15.25	6.29	41.2	“ “

Creatine Content of Filtrates from Lloyd's Reagent.—Folin and Berglund state that treatment with an equal volume of 0.1 N sulfuric acid and with Lloyd's reagent removes all creatinine and creatine. We have found that creatinine is completely removed but that some creatine remains in the filtrate.

Nature of solution.	Creatine per 100 cc.	
	Before.	After.
	mg.	mg.
Normal urine	34.5	11.4
Pneumonia "	64.5	12.1
Pure solution	113.5	20.5

Recovery of Organic Acids Added to Urine.—2 cc. portions of solutions of formic, acetic, lactic, and β -hydroxybutyric acids, respectively, were added to 98 cc. portions of urine. 100 cc. of 0.1 N H_2SO_4 and 30 gm. of Lloyd's reagent were then added.

TABLE I.
Recovery of Organic Acids Added to Normal Urine.

Acid.	Organic acid in urine.	Added.	Titrated.	Recovered.
	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	per cent
Formic.	24.0	16.3	38.1	87
Acetic.	18.7	42.7	59.3	97
"	28.6	42.7	73.1	104
Lactic.	26.6	41.2	67.6	100
"	18.3	41.2	58.8	98
β -Hydroxybutyric.	19.6	57.4*	71.1	89
		54.3†		95

* Calculated from the amount of calcium-zinc hydroxybutyrate taken.

† As determined by titration between pH 8 and 2.7.

After thorough shaking, the mixtures were filtered. The filtrates were shaken with $Ca(OH)_2$ and again filtered. Titration of 50 cc. portions between pH 8.0 and pH 2.7 showed that practically all of the organic acid added was recovered (Table I).

Titration of "Organic Acid" in Normal and Pneumonia Urine.—In Table II are presented the results of the analyses of urine samples by various methods. These were: A, the method of Van Slyke

and Palmer; B, the method of Van Slyke and Palmer, after correction for creatinine and creatine. C, 100 cc. of urine were mixed with 20 cc. of 15 per cent $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and an excess of $\text{Ca}(\text{OH})_2$. After standing 5 minutes, the mixture was filtered and 30 cc. of the filtrate, equivalent to 25 cc. of urine, were titrated in the usual manner. D, 100 cc. of urine were made acid to Congo

TABLE II.
Analyses of Urine Samples by Various Methods.

Subject.	Weight.	Total N.	Organic acid in 24 hrs.				
			Method.				
			A	B	C	D	E
Normal urine.							
	<i>kg.</i>	<i>gm.</i>	<i>cc. 0.1 N</i>	<i>cc. 0.1 N</i>	<i>cc. 0.1 N</i>	<i>cc. 0.1 N</i>	<i>cc. 0.1 N</i>
L	57	7.82	330	225	584	182	91
"		8.05	339	221		240	132
"		7.82	326	208		161	113
M	65	11.8	475	326		336	196
G	75	14.5	629	465	584	382	186
Pneumonia urine.							
M		29.35	1725	1533	1626	531*	326
"		33.83	1501	1324		438	451
"		20.25	914	789		502	398
"		14.18	1009	947		373	364
"		20.53	1040	928		374	286
T		20.78	926	779		478	337
G		30.24	1097	717		500	390
F		29.38	1439	1223		598	457
"		16.03	780	653		319	296

* Analyzed by the method of Van Slyke and Palmer, but with $\text{Ba}(\text{OH})_2$ instead of $\text{Ca}(\text{OH})_2$, 1698 cc.

red with sulfuric acid. Phosphotungstic acid solution was then added until further addition gave no precipitate. The mixture was then diluted to 200 cc., mixed, and filtered. The filtrate was shaken with anhydrous $\text{Ba}(\text{OH})_2$ until just alkaline and was then filtered. 50 cc. of the filtrate were titrated in the usual manner. E, 100 cc. of urine were mixed with an equal volume of 0.1 N H_2SO_4 . 30 gm. of Lloyd's reagent were then added and the mixture

was thoroughly shaken and filtered. The filtrate was shaken with $\text{Ca}(\text{OH})_2$ and filtered. 50 cc. of the filtrate were titrated in the usual manner.

Miscellaneous Data Concerning These Cases of Pneumonia.—Table III summarizes data that may be of interest in the interpretation of the results reported in the preceding portions of this paper. The order of the entries is the same as in the second portion of Table II. None of the urine samples contained more than traces of albumin.

TABLE III.
Miscellaneous Data Concerning These Cases of Pneumonia.

Subject.	Type.	Blood culture.	Date.	Duration of disease.	Temperature.	Partition of N in urine.					
						NH_3	Urea.	Creatinine.	Creatine.	Uric acid.	Undetermined.
						per cent	per cent	per cent	per cent	per cent	per cent
M	V	Positive, Mar. 13.	Mar. 19	11	100	6.74	80.7	1.65	2.68	1.45	6.8
"			" 20	12	100	7.20	81.4	1.49	1.68	1.54	6.7
"			" 21	13	100		89.4	2.02	1.33	1.00	6.6
"			" 22	14	100		88.5	1.18	1.53	1.25	7.5
"			" 23	15	100		90.1	1.71	1.41	1.30	5.5
T	X group.	Negative.	" 23	9	102.6–100		88.6	2.13	2.02	1.35	5.9
G	" "	"	" 10	7	103.8–101.8		88.3	2.37	3.60	0.43	5.3
F	I	Positive, Apr. 11–14.	Apr. 16	11	101–100		90.4	2.10	2.34	1.18	4.0
"			" 17	12	100		88.4	2.10	2.08	0.80	6.6

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LIPID EXCRETION.

VII. THE PARTITION OF FECAL LIPIDS IN BILE FISTULA DOGS.

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In a previous communication (1) four possible sources were suggested for the endogenous lipid excretion which exists in dogs. The possibilities suggested were secretion in the bile, secretion through the intestinal wall, microorganisms, and desquamated epithelial cells. In a later paper (2) data were presented, demonstrating beyond much doubt that bile is not the source. It was found that, when the bile was excluded from the intestine, the excretion increased markedly on a strictly lipid-free diet, whereas, if the bile were the source, it should have disappeared. This finding has been substantiated recently for cholesterol by Beumer and Hepner (3) who fed a cholesterol-free diet to a bile fistula dog and observed a larger excretion than before the operation.

Recently the results were reported of an attempt to obtain information concerning the rôle played by bacteria in this endogenous lipid excretion (4). By a fractional settling process the feces were separated into three fractions (bacterial, non-bacterial, and soluble) and the lipids of each of these fractions were studied. It was found that on the average about 40 per cent of the total lipids was in the bacterial fraction, with the remainder mostly in the non-bacterial fraction. Negligible quantities for the most part were found in the soluble fraction, which should have contained any free lipids or soaps in suspension, and it was concluded from this finding that the excretion was probably contained almost entirely in cellular material, mainly microorganisms and desquamated epithelial cells. The possibility was recognized clearly,

however, that the excretion might represent, in whole or part, a secretion across the intestinal wall which had been adsorbed on solid particles of the feces.

The next logical step appeared to be the determination, by the methods employed in the work discussed in the preceding paragraph, of the partition of the endogenous lipid excretion in bile fistula dogs. It would be very interesting to know the source of the marked increase in excretion, up to 4 times the normal, which takes place when bile is excluded from the intestine. The hypothesis was suggested (2) that there is normally a secretion of lipids into the intestine, which in the presence of bile is largely reabsorbed, but which in the absence of bile appears in the feces as an excretion. If this is true and the normal excretion is contained in cellular material, then the increase in bile fistula dogs should be found largely in the soluble fraction where any suspended free lipids would be. There is no reason to expect any increase in desquamated epithelial cells in a bile fistula dog and it would hardly be likely that an increase in microorganisms large enough to account for the increase in lipids would occur.

EXPERIMENTAL.

*Preparation of Dogs.*¹—The bile fistulas were all of the internal cholecystonephrostomy type as described by Kapsinow, Engle, and Harvey (5). Where collection of uncontaminated bile is not necessary, this preparation has a marked advantage over the conventional external fistula in that no postoperative care is required. There is a rather high percentage of failures due largely to obstruction (particularly high in these experiments, probably due to unfamiliarity with the technique) but on the other hand it is extremely difficult to prevent infection of the bile tract in an animal with an external fistula. For the most part the dogs were in excellent condition during the experiments.² They usually

¹ The author is indebted to Dr. Stabins of the Department of Surgery and Dr. Angevine of this department for performing some of the operations and to various members of this department for assisting with others.

² An autopsy was performed upon Dog 244 at the end of Experiment 7 and it was found that the gallbladder-kidney anastomosis was completely closed. This dog showed very little external evidence of jaundice but the possibility is present that there was more or less stoppage of bile flow throughout the experiments in which the animal was used. Dog 101 was

refused the synthetic diet after a week or two and were then fed by stomach tube.

Diet, Plan of Experiment, and Methods of Analysis.—These were exactly the same as described in the preceding paper (4). More difficulty was experienced however in the fecal separations. It was possible to obtain what appeared to be quite complete separation of feces from normal animals in from ten to fifteen fractionations although of course there were always some non-bacterial particles too small and light to settle away from most of the bacteria and some bacteria too large and heavy to float away from most of the non-bacterial solids. In the case of the feces from bile fistula animals both of these portions seemed to be larger. It was apparent particularly that there was a greater number of large Gram-positive bacteria present than in the case of the normal animals. In some experiments, in fact, there were so many of these large bacteria that a separation was practically impossible. In most cases eighteen or more fractionations were found necessary. The final combined bacterial and non-bacterial fractions were examined carefully under the microscope in each case and an estimate was made of the degree of separation (Table I). These estimates were based on volume (number times average size) rather than on number of particles since most of the non-bacterial particles were much larger than the bacteria. At best they are rather rough approximations, but it is felt that they do give a fairly good measure of the relative degree of separation. Dr. Bayne-Jones of the Department of Bacteriology very kindly examined the fractions from Experiments 1 through 16 inclusive and in general confirmed the opinion of the author.

DISCUSSION.

Comparison with Previous Results.—The results are in complete agreement with those obtained in previous work with bile fistula dogs. In the first sixteen experiments in which small dogs, averaging 4.8 kilos, were used, the average total excretion of lipids was 3.213 gm. per week. In sixteen experiments with

definitely jaundiced during Experiment 16 as evidenced by yellow eyeballs and lips. It is generally recognized that dogs stand jaundice well and the results of the experiments in which there was a question of bile stoppage are in complete agreement with the others in which there was no such question.

normal dogs of just about the same weight (average 4.6 kilos) the average excretion was 1.537 gm. ((4) p. 308). Dog 101, used in both series, excreted an average of 1.509 gm. of total lipids in six experiments while normal and an average of 2.755 gm. in three experiments with bile excluded from the intestine. In the last six experiments heavier dogs, averaging 9.5 kilos, were used and the average excretion was 6.048 gm. Dogs of slightly lower average weight (8.75 kilos) excreted about 2 gm. while normal ((2) p. 366). These experiments emphasize the markedly increased endogenous excretion of lipids by bile fistula dogs. One dog, No. 28-4, was kept on the strictly lipid-free diet continuously for 6 weeks. The condition of the animal was excellent throughout and it was found at autopsy, immediately after Experiment 13, that the bile passage into the kidney was open. As shown in Table I this dog excreted more lipids in the 5th week of the experiment than in the 1st and almost as much in the 6th. The complete lack of relationship between this endogenous excretion and the diet is thus amply confirmed.

The composition of the lipids was found to be essentially the same as in previous work. The average percentage of unsaponifiable material was 27.7. In previous work with bile fistula dogs an average of 31.4 was obtained, while in the experiments with normal dogs reported in the preceding paper (4), where the methods were identical, the value was 29.7. The average values for percentage of fatty acids in total lipids were 64.6, 63.1, and 62.2 respectively in this work, the previous bile fistula experiments, and the preceding work with normal dogs.

Partition of Lipids.—The average percentage of lipids from the bacterial fraction in the total lipids was 48.1; for the non-bacterial fraction the value was 50.9. The soluble fraction, only determined in the first sixteen experiments, contained an average of 1.4 per cent of the total lipids, so it is apparent that, as in the case of the normal dogs, practically all of the lipid excretion, including the excess over the normal excretion, is contained in solid particles which can be removed by settling or centrifuging. At its face value this result would appear to indicate that the increased lipid excretion of the bile fistula dog is due to an increased excretion of cellular lipid material, partly bacterial and partly non-bacterial.

As in the experiments with normal dogs, the composition of the

bacterial fractions with respect to unsaponifiable material and fatty acid content was very similar to that of the non-bacterial. The bacterial fraction contained an average of 30.0 per cent unsaponifiable material and 62.8 per cent fatty acids, while the

TABLE I.
Partition of Lipids on Lipid-Free Diet.

Dog No.	Experiment No.	Total lipids.	"Bacterial" fraction in total lipids.	"Non-bacterial" fraction in total lipids.	Soluble fraction in total lipids.	Bacteria in "bacterial" fraction (estimated).	Non-bacteria in "non-bacterial" fraction (estimated).
		gm.	per cent	per cent	per cent	per cent	per cent
244	1	3.163	24.7	69.8	5.5	90	90
	2	2.648	49.7	49.5	0.8	85-90	80-90
	3	5.848	36.8	61.8	1.4	80-85	50-60
	4	3.737	37.0	62.2	0.8	80-90	80-90
	5	3.401	36.0	61.6	2.4	80-90	80-90
	6	3.681	45.2	52.2	2.6	90	85-90
	7	4.127	48.3	50.4	1.3	90	85-90
28-4	8	2.950	32.5	66.4	1.1	70-80	70-75
	9	2.411	56.8	42.5	0.7	70-75	50 or less.
	10	3.092	68.3	30.7	0.9	75-80	35-45
	11	2.099	51.0	48.4	0.6	75-80	80-85
	12	3.201	47.7	51.4	0.9	70-80	80-85
	13	2.794	41.3	57.5	1.2	60-70	85-90
101	14	2.993	61.3	36.9	1.8	75-80	50
	15	2.068	73.3	26.2	0.5	65-70	85-90
	16	3.205	54.4	45.4	0.2	70-75	80-90
209	17*	7.228	51.8	48.2		65-85	85-90
	18	4.949	37.6	62.4		80-90	85-90
	19	5.198	40.6	59.4		70-80	80-90
186	20	6.537	52.0	48.0		75-90	<60
	21	6.620	53.9	46.1		70-80	90
	22	5.756	57.2	42.8			80-90

Each experimental period represents 1 week. Experiments with each dog represent consecutive weeks except with Dog 244 where there was an interruption of 3 weeks between Experiments 2 and 3.

* Results are estimated owing to partial loss of both bacterial and non-bacterial fractions.

non-bacterial fraction contained 26.4 per cent unsaponifiable and 66.3 per cent fatty acids. In the experiments with normal dogs the values were 29.7 and 67.7 for the bacterial and 29.9 and 63.4 for the non-bacterial fractions respectively. There seems to be

some tendency for a shift of unsaponifiable material to the bacterial and fatty acids to the non-bacterial fraction in these experiments, but it would take more data to establish the point on a statistical basis. Certainly there is very little if any difference in the composition of the two fractions.

So far nothing has been said concerning the effect on the results of the degree of separation of bacteria from non-bacterial substances. A cursory examination shows the complete lack of relationship between the estimated completeness of separation and the content of lipids. Thus in Experiment 10 where 60 per cent of the "non-bacterial" fraction was estimated to be made up of bacteria, the second lowest lipid content was found. In Experiment 14, where the lowest value of all was obtained for the non-bacterial fraction, the separation was quite good. In experiment 3 there was a poor separation of bacteria from non-bacterial substances and here the lipid content of the non-bacterial fraction is high. It is not necessary to pick out further examples; the lack of any correlation is evident from Table I.

The almost complete absence of any lipids in the soluble fraction, where large amounts had been expected, the close similarity of the bacterial and non-bacterial fractions, and the lack of any correlation between the lipid percentage in the two fractions and the degree of separation, all seemed to emphasize the possibility, which was recognized in the preceding paper, that the excretion may represent in whole or part a *secretion* of suspended lipids into the intestine across the intestinal wall, with a subsequent adsorption by various solid particles of the feces. In order to test the possibility of adsorption of lipids by fecal particles the experiment was devised of feeding fat along with the basal diet to bile fistula dogs in such quantity that a portion would escape absorption. Separation and analysis of bacterial, non-bacterial, and soluble fractions of the feces as before should then determine whether fecal particles have any appreciable ability to adsorb lipids under the conditions of these experiments.

Three such experiments were carried out on two dogs which had been used in the lipid-free series. Dog 209 received 5 gm. of lard per day in addition to the standard lipid-free diet and Dog 186 received 2 gm. of lard per day. Dog 209 ate the food voluntarily throughout the two consecutive weeks of the two experiments.

Dog 186 ate well at first but on the 4th day vomited, refused to eat, and appeared sick though there was no apparent jaundice. Consequently the experiment was terminated and Experiment 3 (Table II) represents about 2 to 3 days excretion. The results are given in Table II.

It is evident that a considerable portion of the fat fed escaped absorption. On a lipid-free diet Dog 209 excreted an average of 5.792 gm. When this amount is subtracted, 10.176 gm. are left in Experiment 1 and 20.215 gm. in Experiment 2 on Dog 209. Most of these amounts must have come from the 35 gm. per week which the dog received. Dog 186 excreted an average of 6.304 gm. per week on the lipid-free diet. When the figure for the excretion, given in Table II, is multiplied by 3.0, to put it on the

TABLE II.
Partition of Lipids after Lard Feeding.

Dog No.	Experiment No.	Total lipids.	"Bacterial" fraction in total lipids	"Non-bacterial" fraction in total lipids.	Soluble fraction in total lipids.	Bacteria in "bacterial" fraction (estimated).	Non-bacteria in "non-bacterial" fraction (estimated).
		gm.	per cent	per cent	per cent	per cent	per cent
209	1, lard feeding.	15 968	8 0	91 9	0 1	75-85	80-90
	2, " "	26 007	17 0	82 8	0 2	80-90	80-85
186	3, " "	4 151	18 6	79 6	1 8	80-90	80-90

basis of 1 week (of course this is a rough approximation), and subtracted, 6.149 gm. remain, which must have come from the fat in the diet (14 gm. per week). Furthermore it is evident that practically none of this large excretion was present in soluble or suspended form. It was all adsorbed on solid particles of the feces. Further experiments are not necessary to demonstrate the possibility of adsorption of any fat secretion into the intestine by fecal substances. One positive experiment indeed would establish the possibility; three positive with no negative establish the probability.

It is apparent that most of the excess fat is present in and must have been adsorbed by the non-bacterial fraction. Indeed it is a question whether any of the excess is present in the bacterial fraction. The smallest excretion in the bacterial fraction by Dog

209 on a lipid-free diet was 1.860 gm. and the largest 3.744 gm. The bacterial excretion in Experiment 1, Dog 209, is smaller than the minimum, while in Experiment 2 it is somewhat larger than the maximum. The minimum excretion in the bacterial fraction by Dog 186 was 3.299 gm. In Experiment 3, Dog 186, the excretion ($\times 3$) is 2.315 gm.—definitely smaller. The foregoing considerations certainly point to the conclusion that the bacterial fraction adsorbs none of the excess lipids. On the other hand it should be noted that in Experiments 1 and 2, Dog 209, the absolute amount of unsaponifiable material is markedly lower in the bacterial fraction than in the lipid-free diet experiments. Where the average was 0.689 gm. on a lipid-free diet, it was 0.283 in these experiments. It would seem as if there had been a replacement of unsaponifiable material by fatty acids. The same thing is true but not to so marked a degree in the case of the non-bacterial fraction. It is not true in Experiment 3, Dog 186, but here there was much less excess fat. Obviously there are not enough experiments to warrant definite conclusions on this point.

In summary, the foregoing experiments establish the probability that any fatty secretion into the intestine would be adsorbed mostly on non-bacterial particles of the feces. They do not prove necessarily that the endogenous excretion in normal dogs is such a secretion. It may be and probably is in part at least made up of lipids contained in the cellular material which certainly makes up a large part of the feces; but how much of the excretion is of cellular origin and how much is adsorbed secretion there seems to be no way of saying at the present time. Beumer and Hepner and Bürger and Oeter are of the opinion that the cholesterol portion of the excretion is of secretory origin. Beumer and Hepner have found (3) that the cholesterol in the intestinal contents of a normal dog during absorption is much higher, based on percentage of dried weight, in the colon than in the ileum. They also studied a bile fistula dog after a lipid-free meal and found an even more marked difference, 0.21 per cent cholesterol in dried ileum contents and 1.25 (average) per cent in the colon. They interpret their results to indicate a secretion of cholesterol into the large bowel.

Bürger and Oeter (6) find a definitely greater cholesterol content of the intestinal wall of the sigmoid than of various sections of the

small intestine in cadavers. They interpret this finding to indicate passage of cholesterol across the wall of the large intestine and they have shown that it must be going into the intestine and not out because it contains no coprosterol. Of course selective absorption is possible but not probable in such a low region of the intestine.

SUMMARY AND CONCLUSIONS.

1. The feces of bile fistula dogs on a lipid-free diet have been separated into bacterial, non-bacterial, and soluble fractions, and the lipids have been determined in each of these fractions.

2. The results are in accord with previous data in showing from 2 to 3 times as great a lipid excretion by bile fistula dogs as by normals. One dog continued to excrete lipids at a high level for 6 weeks.

3. As in normal dogs, the excretion was found almost entirely in the solid bacterial and non-bacterial fractions.

4. There was a wide variation in the proportionate amounts of lipids in the two fractions and no correlation was observed with the degree of separation, which in some cases was very poor.

5. The composition of the lipids in the two fractions was very similar, indicating that they came from the same source.

6. When lard was fed to bile fistula dogs along with the lipid-free diet, a considerable amount escaped absorption and this was found almost entirely in the non-bacterial fraction. None was in suspension and the probability is demonstrated, therefore, that any lipids secreted into the intestine would be adsorbed in the same manner.

7. It is not possible to say at the present time what portion of the endogenous lipid excretion is of cellular origin and what part is secreted into the intestine.

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THE SIGNIFICANCE OF CHANGES OF VISCOSITY IN PATHOLOGICAL SERA.

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Measurements of the viscosity of blood serum reported by clinical observers have in many cases shown results that are seemingly paradoxical, as for example a small effect of high dilution on serum viscosity in nephrosis; a low serum viscosity in pernicious anemia, and a high one in icterus, though they are both characterized by a high bilirubin content of the blood; a low serum viscosity in many cases of nephritis and uremia; a very high one in cases of cyanosis. We have attempted to reproduce some of these pathological states in order to investigate the physicochemical basis of these empirical relationships.

The investigations of Loeb (1) on proteins have shown that the measurement of viscosity is an excellent means of demonstrating delicate changes in the physicochemical structure of systems containing large molecules in solution. In blood serum we have a system containing hydrophil colloids which responds to extremely small disturbances in the environment by easily measurable changes in the viscosity.

The viscosity was measured by the time of outflow through a Poiseuille viscosimeter. 10 cc. of blood serum were used and the temperature kept constant within 0.01° at 37.5° in a Freas thermostat. The quotient of the time of outflow of the serum over the time of outflow of pure water through the same viscosimeter at the same temperature was taken as the relative viscosity of the protein solution.

Blood serum, as can be seen from the viscosity at different dilutions (Table I) shows a remarkable ability to keep its viscosity constant in spite of dilution.

If we compare the effects of dilution on the viscosity of such a

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protein solution as gelatin, we find that not alone is the absolute viscosity of the same percentage concentration of serum protein

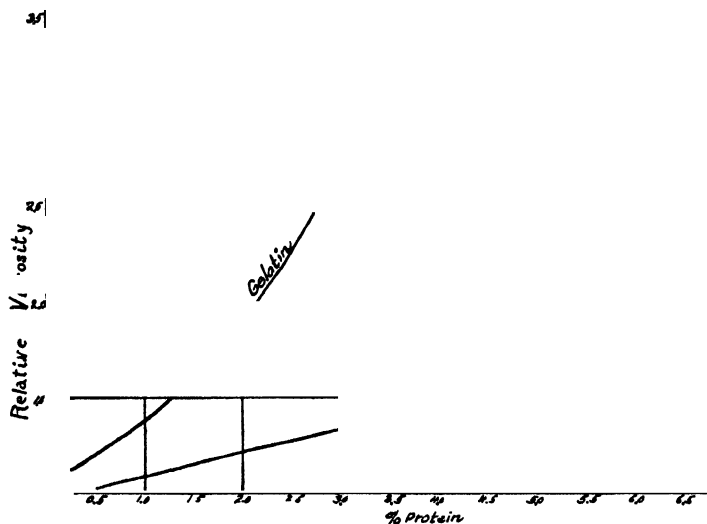


FIG. 1. Relation of viscosity to protein concentration.

very much lower than a similar strength of gelatin, but that the gelatin manifests a much greater change in viscosity for each in-

TABLE I.
Relation of Viscosity to Dilution.

Concentration of serum proteins.		Time.	Relative viscosity.	Relative volume.	$\text{Log } \frac{V}{V_0}$
per cent	gm. per 100 cc.	sec.		per cent	
100	7.	164.8	2 1	44.00	3222
80	5.76	141.8	1.807	32.40	2569
60	4.32	118.4	1.508	20.32	1788
40	2.88	107.4	1.368	14.72	1361
20	1.44	90.0	1.145	5.80	0588
0		78.5	1 0		

creasing unit of dilution (Fig. 1). This point is of great importance in maintaining the circulation of the blood. It would, of

course, be impossible to keep within the circulatory system a protein such as gelatin which would respond to the pathological variations in the concentration of the serum proteins with an enormous change in viscosity. In severe cases of nephrosis, where the blood proteins are reduced to less than 3 per cent, it is obvious that so great a fall in viscosity as would be caused by a protein of the type of gelatin would make the maintenance of the normal circulation impossible.

Einstein (2) has developed a theory of the viscosity of solutions which makes the viscosity a linear function of the relative volume occupied by the solute in the solution.

$$= \left(\frac{V}{V_0} - 1 \right) \frac{100}{2.5}$$

where V_0 is the viscosity of water at the temperature of the experiment, V the viscosity of the solution to be measured, and p the fraction of the volume occupied by the solute in the solution. This formula can only be used when p is very small and the particles of the solute are large in comparison with the molecules of the solvent. This formula enables us to calculate the actual volume of the protein in solution at any dilution. Applied to blood this formula shows that the actual volume of the protein in solution at any dilution is proportional to the dilution itself, and that it is unnecessary to assume any hydration of the protein molecules. Arrhenius' (3) formula, $\log V - \log V_0 = ap$, where a is a constant and V , V_0 , and p have the same significance as above, is of more general application. If this formula can be applied to blood then the curve of the logarithms of the viscosity ratio when plotted against the concentration of the proteins should give a straight line. This we find to be the case.

Loeb has distinguished between two types of protein, one represented by gelatin, and the other in his experiments by egg albumin and in our work by the serum proteins. In the former there is an enormous tendency toward the formation of sub-microscopic particles of solid gelatin which do not allow the free passage of the protein ion. As shown by Proctor and Wilson, the unequal distribution of diffusible ions inside and outside the gel leads to an excess of total and molar concentration of the

diffusible ions inside the gel over that outside, and this makes the osmotic pressure higher inside than outside, and the consequence is the diffusion of more water into the gel and an increase of volume of the solid particles. On the other hand, we have such proteins as albumin which show no tendency at a pH in the neighborhood of neutrality and at body temperature toward the formation of these submicroscopic particles. These show a low viscosity and no tendency to jell.

There are certain exudates, especially pleural exudates, which after standing half an hour or so, become jellified. If Loeb's hypothesis were correct, these exudates should show a higher viscosity than other exudates and transudates that do not jell. We have found such to be the case.

TABLE II.
Viscosity of Exudates.

		Protein con- tent.	Time of out- flow.	Viscos- ity	
		<i>gm per 100 cc.</i>	<i>sec.</i>		
Pleural	exudate. . .	3 41	130 5	1 641	Jelled after $\frac{1}{2}$ hour.
Abdominal	"	2 71	98 58	1 243	Did not jell.
Pleural	"	2 21	117 4	1.475	Jelled overnight
"	transudate. .	1 45	86 8	1 091	Did not jell.
"	"	0 09	80 5	1 012	" " "

It can be seen from Table II that an abdominal exudate of higher protein concentration, that did not jell, had a lower viscosity than another exudate of lower protein concentration that did jell. This would seem to prove Loeb's theory of the presence of submicroscopic particles, in this case probably of fibrin, which cause the solution to manifest a higher absolute viscosity and which make it occlude water.

Those proteins which show no tendency to jell also show very little effect on the viscosity of their solutions through changes in the hydrogen ion concentration. On the other hand, proteins such as gelatin show themselves influenced to the same degree in their viscosity and osmotic pressure by changes in the hydrogen ion concentration. 20 cc. of blood serum were diluted with an equal quantity of Ringer's solution containing enough HCl to give

the required pH. The pH was measured potentiometrically. The viscosity of blood proteins was found to be very insensitive to changes in hydrogen ion concentration. It remains almost constant between the ranges of pH 2.8 and 7.4 (Fig. 2).

In an effort to explain the high viscosity found in the serum of patients suffering from cyanosis, carbon dioxide was passed for half an hour through 20 cc. of blood serum and the viscosity of the solution was measured. After that the cells were put back with the serum and a steady stream of CO_2 passed through for 1 hour

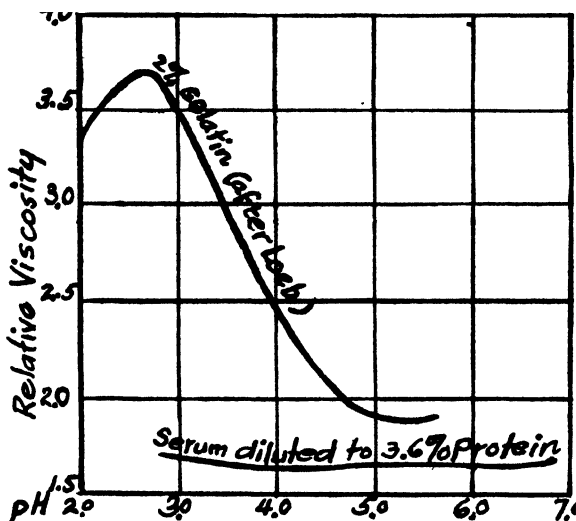


FIG. 2. Relation of viscosity to pH.

with repeated shaking. The cells were then centrifuged quickly and the viscosity of the serum measured. The viscosity was found to have increased (Table III).

Von Limbeck (4) showed that saturating the blood with carbon dioxide causes the cells to swell up with water, which passes into them from the serum. Spiro and Henderson (5) made artificial cells out of collodion membranes and after putting into them solutions of alkali protein salts, they were immersed in isotonic sodium chloride and saturated with carbon dioxide. Bicarbonate was formed in the cells and due to the increase of osmotically active

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HCO_3^- ions in the cells, the latter attracted water from the outside salt solution and increased their volume as do the blood cells.

Inside both cells and serum the positive and negative ions must balance (Van Slyke (6)) and the ratio of chlorion inside and outside the cell must be equal to the bicarbonate ion within and without the cell.

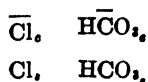
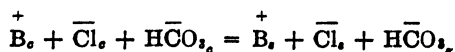


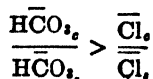
TABLE III.
Relation of Viscosity to CO₂ in Serum.

	Time of outflow.	Viscosity.
	<i>sec.</i>	
Serum.	129.2	1.625
	129.7	1.631
	130.0	1.635
Serum + CO ₂ .	129.7	1.631
	130.0	1.635
	130.1	1.636
Serum + CO ₂ (in presence of cells) after centrifuging off cells.	133.2	1.676
	133.1	1.674
	132.7	1.682

The osmolar concentration obtained by adding the basic ion, the chlorion, and the bicarbonate ion must be equal in serum and cells.



Increase of CO₂ tension lowers the pH_e, causing the base formerly bound by hemoglobin in the cell to shift to the bicarbonate. The increase in bicarbonate ion causes the ratio of the concentration of the bicarbonate ion in the cell to the bicarbonate ion in the serum to exceed the ratio of the chlorion within the cell to that outside.



The increase in $\text{H}\bar{\text{C}}\text{O}_3$ in the corpuscle causes the osmolar concentration in the cell to exceed that in the serum. To restore electrolyte equilibrium, chlorion migrates from serum to cells, and bicarbonate ion in the reverse direction. To restore osmolar equilibrium water migrates from serum to cells until the osmolar concentrations in both are equal. Hence the viscosity of the serum is definitely increased.

In cases of nephritis without edema, we should expect that due to the increase in total solids such as urea, cholesterol, etc., the viscosity of the blood serum would be much increased. Such, however, is not the case.

A study of the cases of Rotky (7) and Austrian (8) shows that their figures are not higher than normal for the viscosity of sera from patients suffering with nephritis. We have found the same to be the case in this laboratory.

We have attempted to find the effect of the addition of cholesterol to serum by shaking serum 1 hour with and without cholesterol and letting it stand overnight. The following morning a determination of the viscosities showed that the serum which had been shaken with the cholesterol showed a lower viscosity (Table IV). Spranger (9) added cholesterol to protein solutions and found that cholesterol increased the viscosity of such solutions. When he added neutral fat (0.5 per cent) and lecithin (0.04 per cent), the viscosity fell on the addition of cholesterol, and still more strikingly on the addition of cholesterol esters. In Ringer's solution containing protein and lipoids in the proportions normally present in blood serum he found an optimum condition for the production of an oil in water emulsion. Very small changes in the ratio of cholesterol to cholesterol esters resulted in the shifting to a water in oil emulsion with a consequent lowering of the viscosity.

The state of solutions containing lipoids and water is dependent on the equilibrium between the emulsions of $\frac{W}{O}$ and $\frac{O}{W}$ (Ostwald (10)). Hydrophil colloids such as albumin, globulin, lecithin, etc., tend to emulsify oil in water. Hydrophobe colloids such as cholesterol tend to emulsify water in oil (Bancroft (11)). With equal amounts of electrolyte and fat, the viscosity must be higher the more particles there are; *i.e.*, the finer the fat is emulsified.

$\frac{W}{O}$ emulsifiers increase the size of the fat particles and cause a fall in the viscosity. $\frac{O}{W}$ emulsifiers split the fat into the smallest particles and bring it into the greatest field of action. This is the reason that the addition of cholesterol to serum, which causes the solution to turn from an $\frac{O}{W}$ emulsion to $\frac{W}{O}$, causes a decrease in the viscosity of the serum. We measured the viscosity of the serum of a rabbit from which 35 cc. of blood had been drawn daily over a period of 2 weeks and found it much lower than the normal serum of a rabbit diluted to the same protein concentration. The serum

TABLE IV.
Relation of Cholesterol to Viscosity of Protein Solutions.

	Concentration of cholesterol.	Time of outflow.	Viscosity.
	mg. per 100 cc.	sec.	
Distilled water.....		79 0	1.00
Human serum.	154	136 2	1.73
“ “ and cholesterol.	198	129.1	1.63
“ “	174	158 0	2.00
“ “ and cholesterol.	209	150.5	1.90
Rabbit “ after repeated hemorrhages..	400	97.96	1.24
“ “ diluted to corresponding protein concentration.	52	111 39	1.41

after the many repeated large hemorrhages showed a fat concentration of over 4 per cent, a cholesterol concentration of 400 mg. per 100 cc., and a protein concentration of 2.84 per cent. In other words, the conditions were those of a $\frac{W}{O}$ emulsion and hence the low viscosity.

It has been known for quite a number of years that the effect of urea has been to decrease the viscosity of water. Because of the physiological and especially the pathological importance of the increase of urea concentration which occurs during the progress of nephritic lesions, the effects of the addition of urea to blood serum were measured. It was found that the viscosity of blood serum

is similarly decreased by the addition of urea (Table V). The decrease is small but definite and on repeated observations outside the limit of error.

It is of some significance that just those blood constituents—cholesterol and urea—which are found higher as a result of kidney lesions, have the power of lowering the serum viscosity. In five cases of uremia, all of which showed abnormally high urea figures, we found, again in agreement with the results of Rotky and Austrian, that the viscosity was fairly normal, due in some measure to the depression in the viscosity caused by the higher urea con-

TABLE V.
Relation of Viscosity to Concentration of Urea.

	Concentration of urea.	Time of outflow.	Relative viscosity.
	<i>gm. per 100 cc.</i>	<i>sec.</i>	
Distilled water.	0	80.0	1.00
		80 1	
Rabbit serum.	0.024	126.0	1.59
“ “ and urea.	1.48	124 4	1.57
Human “	0.024	142 6	1.78
“ “ and urea.	0.48	140.4	1.76
	0.310	138.8	1.74
Serum of patient with uremia.	0.195	151.2	1.89
“ “ “ “ “	0.324	150.1	1.88
“ “ “ “ “ cited by Austrian			1.92

centration, even though there was a great increase in the amount of total solids in the serum.

The sera of patients suffering with pernicious anemia and hemolytic jaundice, though showing an increase in the concentration of bilirubin, do not manifest a higher viscosity. On the other hand, the sera of patients suffering with obstructive jaundice show an enormous increase. The presence of bile acids, whose exact concentration in the blood has been a matter of controversy and which have been found by Tashiro (12) as high as 80 to 100 mg. per cent, would seem to offer an explanation for the differences in the viscosities of the sera of patients suffering with these diseases. The bile acids are known greatly to decrease the surface

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tension and increase the viscosity. We should therefore expect that a blood serum showing a positive direct Van den Bergh reaction will have a much higher viscosity than one showing only a positive indirect reaction. Such we find to be the case, as is shown in Table VI.

TABLE VI.
Relation of Viscosity to Bilirubinemia.

	Total protein.	Time of out-flow.	Viscosity.	Van den Bergh reaction.	
	gm. per 100 cc.	sec.		Direct.	Indirect.
Pernicious anemia.....	6.74	115.3	1.45	Negative.	25
“ “ (average of 5 cases cited by Austrian).....			1.35		
Obstructive jaundice.	6.59	232.9	2.93	Positive, immediate.	60
“ “	7.24	227.4	2.86	“ “	22
Hemolytic “	7.01	141.5	1.78	Negative.	45
Obstructive “ (cited by Austrian).....			2.89		

SUMMARY.

An attempt has been made to find the physicochemical basis of the paradoxical serum viscosities found in certain pathological blood sera.

1. The small change of viscosity of blood serum in nephrosis in spite of the high dilution is due to the special molecular state of the serum proteins.

2. The high viscosity of blood serum in cyanosis is due to the migration of water from the serum into the corpuscles, due to the necessity of maintaining the equilibrium of diffusible ions (Donnan equilibrium) and the equilibrium of osmolar concentration.

3. The low viscosity in kidney conditions may be due to the high cholesterol turning the serum from an $\frac{O}{W}$ into a $\frac{W}{O}$ emulsion.

4. In uremia the effect of the urea lowers the viscosity.

5. In obstructive jaundice the bile acids raise the viscosity, which is low in pernicious anemia and hemolytic jaundice in spite of the high bilirubin content.

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A STUDY OF THE ANTIMONY TRICHLORIDE COLOR REACTION FOR VITAMIN A.

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In 1926 Carr and Price (1) pointed out that a blue color, which did not fade perceptibly in 3 minutes, was produced when a chloroform solution of antimony trichloride was added to a potent cod liver oil, and suggested that the blue color observed was a direct measure of vitamin A activity. Other investigators have examined this test and it is generally considered the most reliable color test so far suggested. Recently there has been considerable controversy as to the reliability and specific nature of the test; therefore a critical study of the antimony trichloride color test has been undertaken in this laboratory.

If the blue color produced by antimony trichloride with a potent oil is a measure of the vitamin content of the oil, a correlation should exist between the color test and the biological test of the oil. The blue color produced should also be proportional to the active substance present and consequently to the amount of any one oil used.

In comparing the colorimetric and biological assay of an oil Rosenheim (2) suggested the use of 20 mg. of cod liver oil as the standard amount, because this amount would restore normal growth to rats on a diet otherwise free of vitamin A. This amount of oil they found to equal 10 Lovibond standard blue units when mixed with 1 cc. of reagent and measured in a layer of 10 mm. Wokes and Willimott (3) have shown with three potent cod liver oils the effect of varying amounts of oil with a given amount of reagent. At higher concentrations they found that the blue color produced was not proportional to the amount of oil used and the flattening of the curves was not the same for different oils. They state that the curves cease to be linear functions above 2 to 3 per cent and therefore amounts of oil taken for the test should give readings not higher than 15 to 20 Lovibond blue units. Norris and Danielson (4) have shown that such concentrations cannot be used as a basis for quantitative comparison of the

factor causing the blue color. They found that at no concentration of a potent oil was the color produced a linear function of the amount of oil used and therefore not proportional to the amount of vitamin A present. A reading of 10 to 20 blue units cannot be compared directly with another oil, but if a sufficiently low value be selected the function approaches a straight line and a comparison can be made which is in close agreement with values determined by biological assay.

Wokes and Willimott (3, 5) state that the reaction consists of a color change of blue through yellow to red; and have shown that the intensity of the observed color varies with the strength of the reagent, the temperature, and the time. The reaction is accelerated when the vitamin is rendered unstable by irradiation and aeration (change by irradiation may be due to ozone); olive oil and oleic acid appear to exert some inhibitory effect upon the application of the color tests; pure olive oil and oleic acid give no blue color and the inhibitory effect may be due to the presence of some organic peroxides which would destroy vitamin A.

In the present work a study was made of the color reaction of the non-saponifiable substances of cod liver oil with a chloroform solution of antimony trichloride, and the effect of various factors upon the color developed.

EXPERIMENTAL.

To obtain the unsaponifiable portion of cod liver oil two methods were used. The first method is similar to that proposed by Takahashi and Kawakami (6). 100 gm. of cod liver oil are added to 200 cc. of 20 per cent alcoholic potassium hydroxide and refluxed on a water bath for 30 minutes. After cooling, 200 cc. of water are added and 150 cc. of petroleum ether (b.p. 30–50°). The mixture is shaken and after it has separated into two layers the water layer is again extracted with petroleum ether. This is repeated twice more and the petroleum ether extractions are collected and washed with water until there is no further tendency to form an emulsion. The petroleum ether extract is concentrated by distilling under partial pressure in an atmosphere of carbon dioxide.

The second method is that of Marcus (7). 6.5 gm. of potassium hydroxide are dissolved in 6.25 cc. of water. The solution while warm is added to 25 cc. of cod liver oil. The mixture is stirred and 0.25 cc. of alcohol added. The mass stiffens while the temperature rises. After cooling, 2.25 cc. of water are added and then the mass is extracted several times with ethylene dichloride. The

second method is much simpler and quicker. At the present time we have not been able to extract the vitamin quantitatively by either method.

To use the extract for color assay either the petroleum ether extract or the ethylene dichloride extract is evaporated almost to dryness under partial pressure in an atmosphere of carbon dioxide and the residue is taken up in chloroform.

The antimony trichloride reagent is prepared by dissolving 30 gm. of c.p. Baker's Analyzed antimony trichloride, which is washed in anhydrous chloroform, in 100 cc. of anhydrous chloroform. The solution is stored in the refrigerator and when used is brought to equilibrium at the temperature of an ice and water bath.

The color tests were carried out according to the technique outlined by Norris and Danielson (4); 0.3 cc. of the chloroform extract or oil is pipetted by means of a serological pipette into a half inch cell, and 3 cc. of the antimony trichloride reagent cooled to 2-4°, added in a rapid stream from a pipette. A reading of the color developed is taken by means of a Lovibond tintometer 30 seconds after beginning the addition of antimony trichloride. The period is timed by a stop-watch. The results given in Tables I to V are averages of three or more separate readings. In the tables B, Y, and R refer to Lovibond standards, blue series 1180, yellow series 510, and red series 200 respectively. In order to show the relationship between the color developed by different concentrations an extract was diluted with chloroform. In each case the original extract is referred to as 100 per cent by volume and the more dilute as 75 per cent, 50 per cent, etc., with regard to the proportion of the original extract and chloroform added.

Table I and Fig. 1 show the results of four distinct extractions of the unsaponifiable portion of cod liver oil, and also the results of using varying amounts of a typical cod liver oil (Squibb). The oil was diluted with chloroform in the same manner as the unsaponifiable extract.

Extracts 3 and 4 were kept in an ice box and samples tested at the end of 2 and 4 months. The values differed by less than 0.2 of a blue unit from the original readings. This would indicate that the blue-producing substance is stable in petroleum ether in the cold and dark.

From Table I and Fig. 1 it will be observed that the extracts of

TABLE I.
Color Produced by Cod Liver Oil and Extracts of Unsaponifiable Portion of Cod Liver Oil with Antimony Trichloride.

Extract No.	Substance.	100 per cent.			50 per cent.			25 per cent.			12.5 per cent.			6.25 per cent.		
		B	Y	R	B	Y	R	B	Y	R	B	Y	R	B	Y	R
1	Extract of unsaponifiable portion (Squibb).	6 6	2 8		3 1	1 3		1 5	0 6		0 8	0 4				
2	Same (Patch).	7 3	3 2		3 7	1 5		2 0	0 6		1 1	0 6				
3	" (").	14 1	5 8		7 3	1 8		3 6	1 4		1 9	0 8		0 9	0 3	
4	" (Squibb).	8 8	3 7		4 4	1 7		2 2	0 9		1 1	0 5				
5	Cod liver oil (Squibb).	14 0	8 8	28 8	14 0		11 8	13 0	1 8	1 8	9 7	2 1	1 4	5 6	0 8	0 8

B, Y, and R denote Lovibond standards, blue series 1180, yellow series 510, and red series 200 respectively. The original extract is referred to as 100 per cent by volume, and the more dilute as 50 per cent, etc.

the unsaponifiable portion of cod liver oil produce no red coloration and that the blue color of the extracts is a linear function of the percentage concentration, while the curve produced with cod liver oil is not a linear function. Work is now being carried on in this laboratory to correlate the biological rat unit with the Lovibond blue unit.

In preparing the above extracts of unsaponifiable portion of cod liver oil, petroleum ether and ethylene dichloride solutions were

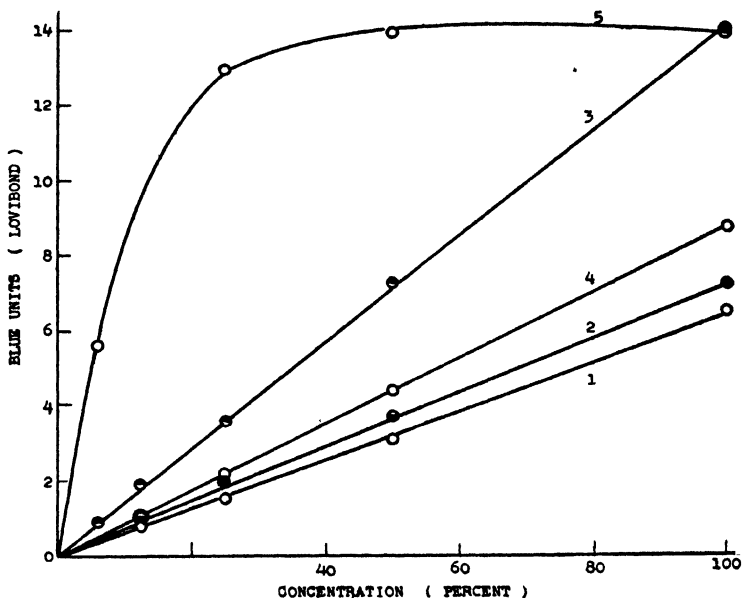


FIG. 1. Intensity of color plotted against the concentration of solution used in the test. Curves 1, 2, 3, and 4 indicate chloroform solutions of the unsaponifiable portion of cod liver oil; Curve 5, cod liver oil (Squibb).

evaporated almost to dryness, the residue being taken up in chloroform. This would give a solution containing traces of the other two solvents. In order to determine whether the small amounts of the above solvents left in the chloroform solution would have any effect upon the intensity of color produced, solutions were prepared containing varying amounts of these and other solvents and color tests made in the usual manner.

In preparing the solutions 1 cc. of a chloroform extract was pipetted into each of four tubes, 0, 1, 2, and 3 cc. of the solvent to be studied added to successive tubes, and each tube diluted to 4 cc. with chloroform; this gives a chloroform solution containing 0, 25, 50, and 75 per cent by volume of the second solvent. U.S.P. reagents were used and also reagents dried over calcium chloride and anhydrous sodium sulfate. The results given in Table II would indicate that traces of moisture have little or no effect upon the development of color. No solvent was found in which a deeper

TABLE II.

Effect of Solvents upon Color Produced by Antimony Trichloride upon Extract of Unsaponifiable Portion of Cod Liver Oil.

Results are expressed in Lovibond units.

Solvent.	0 per cent.		25 per cent.		50 per cent.		75 per cent.	
	B	Y	B	Y	B	Y	B	Y
Petroleum ether	3.6	1.5	3.6	1.5	3.5	1.7	3.3	1.4
“ ether-dried CaCl_2	3.6	1.2	3.6	1.2	3.5	1.3	3.2	1.3
Absolute alcohol	3.6	1.2	3.2	1.5	2.5	1.4	1.1	0.8
95 per cent “	3.6	1.2					1.1	1.1
Acetone-dried CaCl_2	3.6	1.2	3.3	1.4	2.7	1.4	2.1	1.4
Acetone	3.6	1.2					2.1	1.4
Benzene dried over Na_2SO_4	3.7	1.5	3.7	1.4	3.5	1.3	3.3	1.3
CCl_4 “ “ “	3.7	1.5	3.7	1.3	3.5	1.3	3.3	1.3
CCl_4	3.7	1.5					3.3	1.3
Ether-dried Na_2SO_4	3.7	1.5	2.8	1.3	1.2	1.3		
CS_2 -dried Na_2SO_4	3.7	1.5	3.4	1.3	3.5	1.3	3.3	1.3
CS_2	3.7	1.5					3.3	1.3
Ethylene dichloride redistilled ...	3.3	1.3	3.3	1.0	3.3	1.0	3.3	1.0

blue was produced than in chloroform. Petroleum ether and ethylene dichloride had no effect up to 25 per cent by volume, consequently the traces remaining in the extracts previously used would have no influence upon the color. Alcohol, ether, and acetone of the solvents tried decreased the color most markedly, but in no case was there any development of red coloration.

Since the curve for cod liver oil differs so radically from that for the purified unsaponifiable fraction of the same oil, and at the same time the divergence of the concentration curve from a linear function varies with different cod liver oils, it is evident that

some chemical compound common to all oils but varying in amount in different oils, and which is removed by saponification and extraction, must have an inhibitory effect upon the observed blue color. Oils differ in free fatty acids, oxidation products, and impurities, depending upon the process of preparation and purification.

The effect of various saturated and unsaturated free fatty acids upon the intensity of the blue color produced by the action of the unsaponifiable portion of cod liver oil and chloroform solution of antimony trichloride was measured by setting up series of tubes, each containing an equal amount of a chloroform extract of the unsaponifiable portion of cod liver oil, and adding to successive tubes varying amounts of chloroform solutions of fatty acids such that the final concentrations of fatty acids should be 0, 7.5, 12.5, 25, 50, and 75 per cent when each tube was diluted with chloroform to the same volume. If the fatty acid has any appreciable effect upon the color produced, the measured color value will vary from the standard or 0 per cent. The results given in Table III show that the saturated fatty acids have no effect upon the color observed. It is more difficult to obtain pure unsaturated fatty acids; of those used Δ^6 -hexenic, crotonic, and cinnamic acids showed only a slight inhibiting effect on the development of a blue color, while U.S.P. oleic acid showed a very marked decrease in blue with increasing concentration and the rapid development of a red coloration. The saturated and other unsaturated fatty acids used showed absolutely no red coloration with the exception of a very light color, 0.4 red units, with 50 per cent crotonic acid.

The oleic acid used in Table III was of U.S.P. grade and of yellow color; as the hexenic acid had very little effect upon the color produced it was probably not the unsaturated fatty acid itself but some impurity or oxidation product which caused the red color. The purest oleic acid obtainable on the market (free from linoleic acid) had a slight straw color and gave a very marked red with antimony trichloride. Effort was made to purify oleic acid both by its lead salt and by fractionally distilling several times under reduced pressure in an atmosphere of carbon dioxide. A colorless product was obtained which gave only a very slight pink color when first mixed with antimony trichloride reagent, but developed into a deep red. Bubbling oxygen through oleic acid for 2 hours at room

TABLE III.
Effect of Various Fatty Acids upon Color Produced by Antimony Trichloride with Extract of Unaponifiable Portion of Cod Liver Oil.

Results are expressed in Lovibond units.

Fatty acid.		Per cent of fatty acid in final mixture.																				
		0						7.5			12.5			25.0			50.0			75.0		
		B	Y	R	B	Y	R	B	Y	R	B	Y	R	B	Y	R	B	Y	R			
1	Propionic.	4.7	1.8																			
2	Butyric, b.p. 160-165°.	7.7	3.2																			
3	Isobutyric, b.p. 155-160°.	7.7	3.2																			
4	Lauric.	7.5	3.3																			
5	Myristic.	7.5	3.3																			
6	Palmitic.	7.5	3.3																			
7	Stearic.	7.5	3.3																			
8	Δ^{α} -Hexenic.	7.5	3.1																			
9	Crotonic.	11.5	4.8																			
10	Cinnamic.	11.5	4.8																			
11	Oleic (U.S.P.).	5.3	2.1																			

temperature had no effect upon the color produced when mixed with a solution of the unsaponifiable fraction of cod liver oil and added to antimony trichloride reagent.

Saturated oils as coconut oil, even when strongly rancid, give no color with antimony trichloride reagent, while unsaturated oils, as olive, peanut, and linseed, give varying degrees of deep red but no blue color. The effect seems not to be due to oxidation to a single hydroxy group, as ricinolein (castor oil) gives only a very slight pink. The results produced by an oil as olive oil when mixed in varying proportions with a fixed amount of the unsaponifiable portion of cod liver oil, Table IV, might be explained on the basis

TABLE IV.

Effect of Olive Oil upon Color Produced by Antimony Trichloride with Extract of Unsaponifiable Portion of Cod Liver Oil.

Results are expressed in Lovibond units.

Olive oil.	Color produced.			Decrease of blue.
	B	Y	R	
<i>per cent</i>				<i>per cent</i>
0.0	7.5	3.2		0.0
3.12	5.7	1.9		24.0
6.25	5.5	2.1		26.7
12.5	5.2	2.0		30.6
25.0	4.7	2.0	0.3	37.3
75.0	3.7	1.8	0.3	50.6

of the free fatty acid present. The olive oil used had the following constants: iodine number (Hanus) 80.7 and acid number 0.799.

Oleic acid and unsaturated oils show a very marked inhibiting effect upon the blue color when measured according to the technique of Norris and Danielson, at 30 seconds after mixing the solutions. However immediately upon mixing a solution of the unsaponifiable portion of cod liver oil containing oleic acid or unsaturated oils with antimony trichloride reagent, a blue flash lasting in the higher concentrations of oleic acid only a very short period of time was observed, the blue color fading very rapidly. This would indicate that the oleic acid or an impurity in the acid did not destroy the blue-producing substance nor absolutely inhibit the formation of a blue color but accelerated the rate of fading of the

blue. Table V gives the results of tests showing the effect of oleic acid (Eimer and Amend, c.p., free of linoleic) upon the rate of fading of the blue color and the development of a red coloration. The rate of fading of the blue is greatly increased by increasing amounts of oleic acid used, so that readings taken at 30 seconds vary greatly in the measured intensity of blue although the amount of blue-producing substance in each case is known to be the same. Oleic

TABLE V.

Effect of Oleic Acid upon Rate of Color Development of Extract of Unsaponifiable Portion of Cod Liver Oil with Antimony Trichloride Reagent.

Results are expressed in Lovibond units.

Time.	Per cent oleic acid.														
	0.0			6.25			12.5			25.0			50.0		
	B	Y	R	B	Y	R	B	Y	R	B	Y	R	B	Y	R
<i>sec.</i>															
5													5.7	4.7	3.7
10	8.5	2.6		7.9	2.8		7.4	2.1		5.7	2.7	1.1	3.4	3.3	4.1
20	8.4	2.6		7.2	2.2					4.5	2.3	2.5			
30	8.3	2.6		6.6	2.2	0.3	4.9	1.8	0.6	4.4	2.8	3.6	2.9	4.0	7.2
<i>min.</i>															
1	8.1	2.6		5.7	2.2	0.9	4.5	2.0	1.9	3.7	2.4	4.7	2.7	4.7	10.3
2	7.7	2.4		4.5	2.2	1.8	3.1	2.0	2.9	2.9	2.4	5.8	2.7	4.7	12.0
3	6.7	2.1		4.1	2.2	2.2	3.2	1.8	3.3	2.7	2.5	5.6			
4	6.1	1.8		3.7	2.0	2.6	2.7	1.8	3.5	2.4	2.7	6.1			
5	5.1	1.4		3.3	1.9	2.6	2.4	1.8	3.5	2.3	2.7	6.3			
6	4.3	1.1		2.5	1.7	2.6	2.3	1.8	3.6	2.0	2.7	6.4			
7	3.8	1.1	0.1	2.8	1.7	2.6	2.3	1.8	3.7	2.2	2.9	6.5			
8	3.7	1.1	0.3	2.6	1.7	2.6				2.1	2.9	6.6			
9	3.6	1.1	0.4												

acid without the extract of the unsaponifiable portion of cod liver oil shows a development of red color reaching a maximum with each concentration of acid similar to the red values obtained when oleic acid is added to the extract of unsaponifiable substance of cod liver oil. Therefore it would seem that the color change for vitamin A, if the color be due to the vitamin, is not one from blue through yellow to red as suggested by Wokes and Willimott but the substance producing the "blue" color produces a greenish blue with a ratio of blue to yellow of approximately 1:0.4 when meas-

ured in Lovibond units, and the color fades out to a colorless solution. The red coloration is produced by some other substance present in oleic acid and unsaturated oils.

In attempting to determine the vitamin A by the intensity of the blue color produced by cod liver oil with a chloroform solution

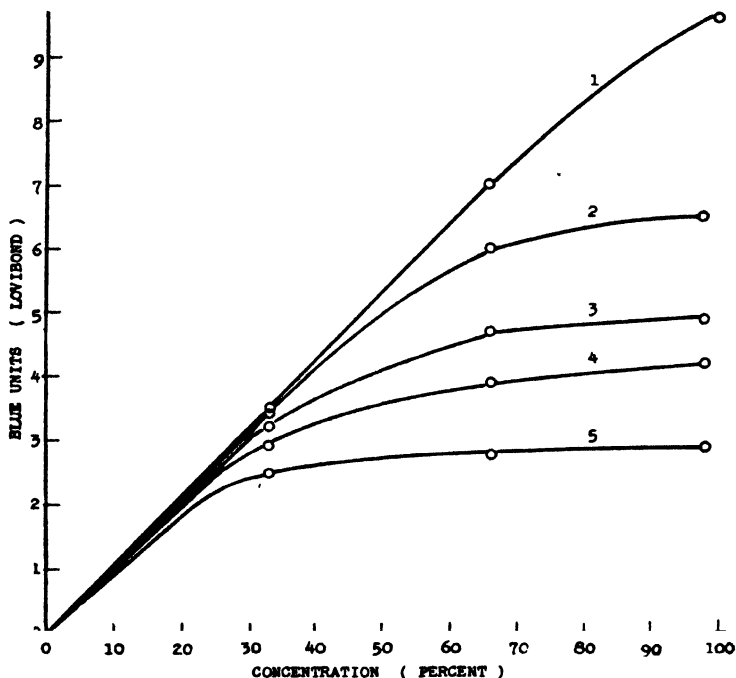


FIG. 2. The effect of oleic acid upon the intensity of color produced plotted against the concentration of the solution used in the test. Curve 1 indicates a chloroform solution of the unsaponifiable portion of cod liver oil; Curve 2, the same containing 6.25 per cent oleic acid; Curve 3, the same containing 12.5 per cent oleic acid; Curve 4, the same containing 25.0 per cent oleic acid; Curve 5, the same containing 50.0 per cent oleic acid.

of antimony trichloride, two variables must be considered, the first the amount of vitamin A or blue-producing substance and the second that which increases the rate of fading of the blue and probably also causes the red coloration. The results obtained with known but varying amounts of these two factors are given in Fig. 2. Tubes were set up in three series such that each tube of a

given series would contain the same amount of the unsaponifiable extract of cod liver oil producing a clear greenish blue and successive tubes of the series to have a final concentration of 0.0, 6.25, 12.5, 25.0, and 50.0 per cent oleic acid. The amount of unsaponifiable portion of cod liver oil in the first series is designated as 100 per cent, the second series contained two-thirds of the first, and the third series one-third of the first. Color tests were made according to the technique of Norris and Danielson, readings being taken at 30 seconds. Fig. 2 gives the data (the values for yellow and red being omitted) calculated to represent the effect obtained with five oils each having exactly the same amount of vitamin A but differing from one another in the amount of oleic acid or red-producing substance present in the initial oil, the initial oils each being diluted to two-thirds and one-third by volume with chloroform, and color tests made upon the resulting solutions. As the amount of oleic acid increases, the divergence of the curve from a linear function becomes greater; 100 per cent of the initial oils known to contain the same amount of vitamin A is seen to give color values varying from 2.9 to 9.6 blue units. At the lower color values as the oils become more dilute the interference of the red-producing substance becomes less marked; the curves approach more closely a linear function so that the color observed for a definite known vitamin content checks more closely in the several oils. With 12.5 per cent oleic acid the color for the oil (4.9) is approximately 50 per cent of that with no oleic acid; when diluted to one-third with chloroform, the observed value is 3.2 blue units, within 10 per cent of that without oleic acid (3.5). Therefore with the ratio of the interfering substance to vitamin A present to the extent that would be represented by 12.5 per cent of the oleic acid used, if the comparison were made below a color value of 3.0 blue units the results would vary by less than 10 per cent; if the comparison were made above 4 blue units the error would be as great as 50 per cent. These results substantiate the findings of Norris and Danielson that at sufficiently low color values the curves for oils approach linear functions and a comparison can be made between the colorimetric and feeding experiments. However the color tests must be very carefully controlled as to time, temperature, and concentration of the reagent. The tests are best made in a constant temperature room; if tests be made on a single oil at various

times during the course of an experiment extending through different seasons so that the room temperature will vary by several degrees, observed values at later dates may be greater or less than the initial observed value depending upon whether the room temperature be cooler or warmer.

SUMMARY.

1. Vitamin A, or the substance producing the "blue" color with antimony trichloride reagent, produces a greenish blue which fades to a colorless solution; the ratio of blue to yellow is approximately 1.0 to 0.4 Lovibond unit.

2. The blue color produced by an extract of the unsaponifiable portion of cod liver oil with a chloroform solution of antimony trichloride is a linear function of the percentage concentration of the extract, and no red coloration develops on standing.

3. Traces of petroleum ether and ethylene dichloride have no effect upon the intensity of the color produced.

4. Saturated fatty acids and oils have no effect upon the color produced by the unsaponifiable portion of cod liver oil with antimony trichloride reagent.

5. Oleic acid and unsaturated oils accelerate the rate of fading of the blue color.

6. Deviation of the observed blue color produced with varying amounts of cod liver oil from a linear function is due to an increased rate of fading of the blue color.

7. Quantitative comparison of the color values between different oils or between colorimetric and feeding experiments can only be made at a sufficiently low value so that the dilution curve approaches a linear function, or be made on the unsaponifiable portion.

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ON THE DETERMINATION OF DIFFUSIBLE AND NON-DIFFUSIBLE SERUM CALCIUM.

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INTRODUCTION.

Ultrafiltration offers an empirical means of determining the calcium distribution in the blood. The same objections that apply to ultrafiltration also apply to compensation dialysis in that most probably neither method gives the actual ionized and non-ionized calcium present in blood plasma. It is interesting to note in this connection that the dialysis experiments, *in vivo*, of Stewart and Percival (1), in which the living peritoneal cavity is employed as a dialysis sac, yield about the same values for diffusible calcium as are obtained by the ultrafiltration method. The analysis of cerebrospinal fluid also yields similar results (2). Because of its comparative simplicity and rapidity, ultrafiltration offers the readiest means of determining the distribution of calcium in blood serum. The results obtained by this method have been consistent and offer results of readily comparative value, even if perhaps they are at present not completely theoretically interpretable.

Ultrafiltration as a means of determining the diffusible calcium of blood serum, with large samples of blood, has been employed by many investigators among whom may be mentioned Cushny (3), Neuhausen and Pincus (4), Richter-Quittner (5), and Pincus, Peterson, and Kramer (6). Moritz (7) developed a micro method for determining diffusible calcium, employing a combination of ultrafiltration and diffusion. The method of Moritz was improved in some respects by Updegraff, Greenberg, and Clark (8). Since

then a number of studies in which use is made of this micro method have appeared by Lui (9) and Reed (10).

In the course of the recent studies on blood calcium carried out by us, a number of improvements have been made in the technique of the ultrafiltration procedure and certain experiments carried out having both a practical and theoretical bearing on the determination of the true distribution of the diffusible and non-diffusible calcium of the serum. In connection with the improvements in technique, a system of analysis has been developed for total calcium, diffusible calcium, inorganic phosphate, and serum proteins on the serum from about 12 cc. of blood, with the use of the colorimetric method for serum proteins and the supernatant fluid from calcium analysis for phosphate determination already published by us (11, 12).

EXPERIMENTAL.

Ultrafiltration Procedure.—For determining diffusible calcium, ultrafiltration alone is now used by us to obtain a protein-free filtrate. No water or aqueous solution is placed in the outside container in contact with the collodion membrane. We have abandoned the combination of ultrafiltration and diffusion to eliminate any doubt that diffusion equilibrium may not be established between the serum in the collodion sac and the external solution in the course of time of the Moritz procedure (7), and also to avoid possible changes in equilibrium between diffusible and non-diffusible calcium. This subject will be considered again in connection with the subject of the nature of the equilibrium between diffusible and non-diffusible serum calcium.

The apparatus used is essentially the same as that described by Updegraff, Greenberg, and Clark (8). However, instead of using the mercury syphon illustrated in Fig. 2 of that publication, to maintain the external reduced pressure required for filtration, use is now made of a water- or motor-driven vacuum pump. The mercury syphon required almost constant attention to keep the pressure difference at the required level. By the use of a vacuum pump and a regulating valve, the proper pressure is maintained automatically for any desired length of time. A regulating valve that is very satisfactory for the purpose is quite easily made. A glass cylinder of 200 mm. or more in height is fitted with a 3-hole

rubber stopper; two bent glass tubes as shown are used to connect between the vacuum pump and the rest of the apparatus with rubber tubing; a third straight piece of capillary tubing of about 1 mm. bore is inserted through the third hole into the mercury contained in the cylinder, to a depth of 150 mm. below the surface of the mercury. This automatically keeps the vacuum at 150 mm. of Hg below atmospheric pressure when a water pump is employed.

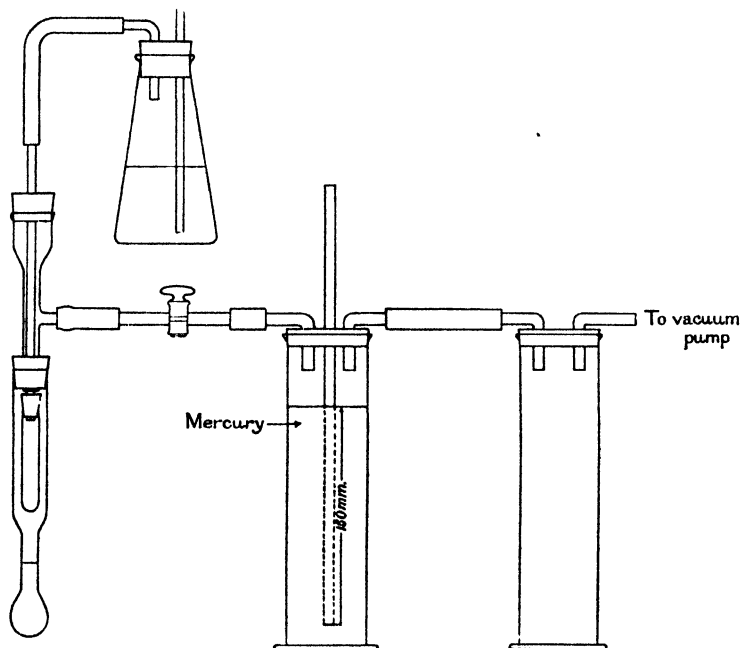


FIG. 1. Diagram of ultrafiltration apparatus with an automatic pressure-regulating cylinder.

By accurately setting the regulating tube, a manometer to show the pressure difference can be dispensed with. It is also desirable to have a wash bottle between the regulating cylinder and the pump to trap any mercury splashed over, or any water sucked back from the pump. When it is desirable to measure the amount of ultrafiltrate that comes through, the filtration apparatus is fitted into a Folin sugar tube with dimensions of 2.5 cm. inside diameter and 19 cm. long, having a constricted portion of 5 cc.

at the bottom. In Fig. 1 there is shown a sketch of such an ultrafiltration apparatus with a manometer omitted.

Collodion Solution.—The selection of a satisfactory collodion solution is of paramount importance for obtaining correct ultrafiltration results. The parlodion solution containing olive oil, introduced by Moritz, has been abandoned by us because it does not give uniformly permeable membranes. We have tried out various solutions made from parlodion, Merck's and Schering's collodion. Parlodion was found to give a slower rate of filtration than the other two mentioned, which were about equal in this respect. No one of the three collodions in simply alcohol-ether solution gave sacs, however, that were uniformly completely permeable to calcium chloride. To obtain completely satisfactory membranes, we found it necessary to add either glycerol or ethylene glycol to the alcohol-ether collodion solution (13). About 5 per cent of glycerol or ethylene glycol added to parlodion or Schering's collodion was found to give suitable membranes, although those with glycerol had a tendency to be weak. Collodion membranes with ethylene glycol were considerably stronger. Our final choice was a solution of 7 per cent Schering's collodion in 60 parts of ether and 40 parts of absolute alcohol, to which there are added 5 cc. of ethylene glycol per 100 cc. of collodion solution. This solution proved to be completely permeable to calcium and to hold back the serum proteins completely. Each stock of collodion solution prepared is tested by us for permeability by filtering a calcium-containing solution, with a calcium concentration of about 10 mg. per 100 cc. through a number of sacs. The sacs are cast as described by Updegraff, Greenberg, and Clark (8), with two dippings of collodion solution. After tying on the collodion sac to the rubber stopper of the filtering apparatus, it has been found useful to seal the sac to the stopper by painting it with a little collodion solution. This makes a leak-proof seal when the collodion dries.

A necessary precaution in carrying out the ultrafiltration is to wipe off the first few drops of fluid that appear on the outside of the sac the first few minutes after filtration is started. This represents the displaced capillary water of the collodion sac. If this is not done, the values obtained for diffusible calcium will be a little low, as has been shown by Neuhausen and Pincus (4). To determine

the magnitude of the dilution possible due to capillary water, in the size of the membrane employed by us, a test run was carried out. It was found that the same sample of serum gave a 6.5 per cent lower value for diffusible calcium in a sac left unwiped than in one that was wiped when 4 cc. of serum were introduced into the sac and about 2 cc. ultrafiltered. As a still further test, four cast sacs were wiped dry with filter paper, weighed, dried overnight in 105° in an electric oven, and the loss in weight determined. The loss of weight was found to be 0.745 gm. for the four sacs or an average of 0.186 gm. per sac. This indicates that there are less than 0.2 cc. of water contained in the pores of a membrane sac. With the 3 or 4 cc. of serum employed for ultrafiltration by us, this amount of water cannot cause more than a 10 per cent dilution, even if the first extruded drops are not wiped off.

Analytical Methods.

For calcium, the Tisdall modification of the Kramer-Tisdall method is employed except that precipitation is allowed to continue for at least 2 hours before centrifuging (14). In washing the calcium oxalate precipitate, a 2 per cent ammonia solution saturated with calcium oxalate is used, as recommended by Stanford and Wheatley (15). Samples containing as much calcium as is usually found in 2 cc. of serum are titrated with 0.005 N potassium permanganate that is freshly diluted as needed from a 0.05 or 0.1 N stock solution. With less amounts of calcium, as in the samples of the ultrafiltrate for diffusible calcium, the Van Slyke and Sendroy gasometric method (16) is used to measure the amount of calcium oxalate precipitate. We found about 5 minutes shaking was required for complete evolution of the CO₂, rather than the 3 minutes stated by Van Slyke and Sendroy. In the communication of Updegraff, Greenberg, and Clark ((8) p. 95) it was pointed out that in the ultrafiltrate samples films of calcium oxalate very often remained floating on the surface after the tubes had been centrifuged. To avoid such films, we have adopted the procedure of adding 1 cc. of supernatant fluid from the determination of total serum or previously decalcified serum in place of an equivalent amount of distilled water in setting up the tubes for analysis.

The determination of inorganic phosphate is made by Fiske and Subbarow's method on serum (17) or on the supernatant fluid from calcium analysis (12), and colorimetric analysis for serum proteins was carried out as previously mentioned (11).

System of Analysis.—About 12 cc. of blood are drawn for the purpose of this analysis. After separating the serum, 2 cc. of the serum are pipetted out for total calcium analysis, 0.5 cc. for protein analysis, and the remainder is used for ultrafiltration. The inorganic phosphate is determined in the supernatant fluid from the total calcium determination. In the procedure there is no need to measure carefully the amount of serum used for ultrafiltration. The ultrafiltration is allowed to go on for from 3 to 4 hours. In that time approximately half the serum introduced in the collodion sac is ultrafiltered. The calculations involved are straightforward. Each sample taken represents the conditions of the original serum except for a correction for the volume of the serum proteins which are, of course, absent from the ultrafiltrate liquid.¹

¹ If less serum is available, the complete analysis can be carried out on as little as 3 cc. of serum by following this procedure, although the results obtained are subject to greater errors. In the alternate method, 3 or 4 cc. of serum are accurately pipetted into a collodion sac that has been wiped dry. After the collodion sac has been fitted onto the rubber stopper, the filtration apparatus is fitted into a Folin sugar tube with dimensions of 2.5 cm. inside diameter and 19 cm. long, having a constricted portion of 5 cc. at the bottom (see Fig. 1). This conveniently allows the determination of the amount of ultrafiltrate that has come through, by measuring the amount of water required to fill the 5 cc. portion. A micro burette graduated in 0.02 cc. is very convenient for this purpose.

At the end of the filtration, 4 cc. of the fluid containing the ultrafiltrate are measured into a 15 cc. centrifuge tube and 1 cc. of 4 per cent ammonium oxalate added for diffusible calcium analysis. The supernatant liquid after centrifuging is poured off to be used for inorganic phosphate determination. On the *concentrate* left in the collodion sac there are determined proteins and calcium. The contents of the sac are transferred by puncturing the end to the constricted portion of one of the above Folin sugar tubes, the top of which has been cut off a few cm. above the constricted portion. The concentrate is made up to 5 cc. by filling to the 5 cc. mark with water. A 3 cc. or other convenient aliquot is taken for calcium analysis. The serum proteins are determined on a 0.5 cc. portion in the usual manner.

Calculation.—For the protein results it is only necessary to remember that all the protein is retained in the collodion sac. If a 3 cc. sample has been used, the 0.5 cc. aliquot from the Folin tube represents 0.3 cc. of serum. The rest of the calculation is as given in the formula ((11) p. 549).

Experiments on Interpretation of Calcium Partition.

Effect of Time of Contact of Serum with Clot on Serum Calcium.—It is important to know whether after the clot is once formed there is any change in the amount or the distribution of the calcium in the serum if the clot is allowed to remain in contact with the serum. Aside from the theoretical implications of this problem, we were interested from the practical point of deciding when best a separation of serum from blood could be made. Blood that is centrifuged immediately after clotting does not give a separation of as much serum as blood that has stood sufficiently long for the clot to retract. The subject also concerned us because many of our samples, coming from San Francisco to Berkeley, required some 6 hours to be brought for analysis. A number of experiments, therefore, were carried out to determine the effect of time of contact of serum with the clot. Freshly obtained beef blood was separated into a number of fractions which were centrifuged and the serum separated from the clot at varying intervals of time. The total calcium and, in one series, the diffusible calcium were determined on these serum fractions. All analyses were performed in duplicate. It took $\frac{3}{4}$ of an hour after the blood was obtained to reach the laboratory and to start centrifuging the first sample. The blood, on reaching the laboratory, was still warm. It was found, as is shown in Table I, that the calcium in the serum is independent of the time of contact with the blood clot.

Effect of Carbon Dioxide Tension on Calcium Distribution.—Theoretically, it is to be expected that diffusible calcium will increase with lowered pH and decrease with increased pH of blood

For the calculation of the calcium fractions, the results are calculated to mg. per 100 cc. of *filtrate* and *concentrate*, respectively. Since the calcium present originally in the sac is the sum of the calcium in *concentrate* plus *filtrate*, the total calcium is given by the following equation: $Tca = \frac{a-x}{a} Cca + \frac{x}{a} Fca$; Tca , Cca , and Fca represent total calcium, concentrate calcium, and filtrate calcium, respectively, in mg. per 100 cc. or other convenient unit; a is the cc. of serum introduced into the sac, and x is the amount ultrafiltered. Diffusible calcium is calculated in the usual way from the analysis of the filtrate, and non-diffusible calcium is obtained by difference from the total calcium calculated by the above equation.

TABLE I.
Effect on Serum Calcium of Time of Contact of Serum with Clot.

Serum sample No.	Time of centrifuging.	Total Ca. <i>mg. per 100 cc.</i>	Diffusible Ca. <i>mg. per 100 cc.</i>
1	11.30 a.m.	9.25	5.35
	5.30 p.m.	8.90	5.35
	10.00 a.m.	9.30	5.35
	following day.		
2	9.30 a.m.	9.90	
	1.00 p.m.	9.85	
	4.30 "	9.85	
3	11.00 a.m.	7.65	
	5.00 p.m.	7.70	
	10.00 a.m.	7.65	
	following day. 6.00 p.m.	7.65	

TABLE II.
Effect of pH As Produced by Changes in Carbon Dioxide Tension on Calcium Distribution of Serum.

Experiment No.	Treatment of serum.	pH when put in ultrafiltration sac.	Total Ca. <i>mg. per 100 cc.</i>	Diffusible Ca. <i>mg. per 100 cc.</i>
1	Original serum.	7.4	9.9	3.3
	Some CO ₂ removed by evacuation.	About 8.0	9.9	3.55
	CO ₂ bubbled into serum.	" 7.0	9.9	3.5
2	Original serum.	7.2	10.4	6.65
	Some CO ₂ removed by evacuation.	>8.0	10.4	6.1
	CO ₂ bubbled into serum.	<7.0	10.4	7.1
3 A	Original serum.		8.1	4.05
	Some CO ₂ removed by evacuation.	About 8.0	8.1	4.0
	CO ₂ bubbled into serum.	" 7.0	8.1	4.0
3 B	Original serum.		8.0	4.0
	Some CO ₂ removed by evacuation.	About 8.0	8.0	4.0
	CO ₂ bubbled into serum.	" 7.0	8.0	4.1
4	CO ₂ tension not kept constant.....			4.95
	" " kept constant.....			4.90
5	" " not kept constant.....			5.35
	" " kept constant.....			5.30

serum. However, experiments by von Meysenbug, Pappenheimer, Zucker, and Murray (18) and Neuhausen and Pincus (4) on changing the pH by changing the carbon dioxide tension, showed no appreciable changes in the values of diffusible and non-diffusible calcium. Owing to the theoretical expectations, there is a strong tendency to doubt the results of these authors.

To check for ourselves the effect of pH on the calcium distribution and to make sure that we were justified in neglecting this factor, a number of experiments were carried out on the effect of changes in carbon dioxide tension. The results are summarized in Table II. In Experiments 1 to 3 beef serum was divided into three fractions; one of the fractions was left untreated, CO₂ was bubbled through one portion until the pH was about 7.0, and the third was placed in a vacuum and CO₂ extracted until the pH became about 8.0. The three fractions were then ultrafiltered and the calcium distribution determined. In all the experiments except No. 2, the resulting calcium distribution is independent of the treatment. In Experiment 2 there is a decrease in the diffusible calcium of the evacuated fraction and an increase in the saturated fraction that is outside of the experimental error. This is perhaps due to the evacuation and saturation with carbon dioxide having been carried out to a greater degree in this particular experiment. In Experiment 3 the serum was divided into two portions and then each portion was independently separated into three fractions and treated as described above. The analytical results of the two portions are in very good agreement.

Still another experimental procedure was devised to test the effect of carbon dioxide tension. By the simple procedure of putting some sodium bicarbonate into a phosphate buffer solution of pH 7.4 (0.1 gm. of sodium bicarbonate to 100 cc. of 0.2 M buffer solution), a solution is obtained with a carbon dioxide tension of the same value as that of normal blood. By putting such a solution in the saturating bottle shown in Fig. 1 and connecting to the ultrafiltration apparatus, the filtering serum is maintained at the carbon dioxide tension of the buffer solution throughout the filtration.² Experiments 4 and 5 of Table II give the results of comparing the calcium distribution of pooled human serum

² Because of the simplicity, we are now using such a solution as a regular part of the ultrafiltration procedure.

filtered in connection with the carbon dioxide tension-regulating solution and without. The results show that the same values of diffusible calcium are obtained by either procedure.

The results of Table II substantiate the claim that changes in carbon dioxide tension corresponding to the pH range of about 7 to 8, do not influence the values of diffusible and non-diffusible calcium.

The question arises as to the reason for this seeming independence of calcium distribution on the reaction of the serum. This question for the present cannot be completely or definitely answered. The work of Marrack and Thacker (19) and of Loeb and Nichols (20) indicates, as will be more completely discussed in a following section, that the diffusible calcium, non-diffusible calcium, and the protein content of the serum are functionally related. From the results obtained, it must be concluded that either over the pH range of 7 to 8 there is practically no change in the amount of calcium bound by the serum proteins, or that changes in calcium accompanying changes in reaction are very slow.

Theory of Ultrafiltration.—An understanding of what takes place in the ultrafiltration process is a necessary preliminary to evaluating properly the significance of the values of diffusible calcium obtained by this procedure. Ultrafiltration is an irreversible process and in general there should be a continuous change in the composition of the ultrafiltrate liquid as the colloidal constituents become more and more concentrated. With blood serum, as water and electrolytes are removed by ultrafiltration, the proteins and non-colloidal constituents in combination with the proteins become more concentrated. The ionic strength (21) of the residual solution becomes increased in proportion to the effect of increasing protein. With changes in ionic strength, there are to be expected changes in the equilibrium between diffusible and non-diffusible colloidal constituents.³

³ A number of authors have claimed, and the statement is again brought up by Stewart and Percival (1), that the amount of pressure applied in ultrafiltration influences the value of diffusible calcium obtained by tending to break up labile compounds. This point of view is not well grounded. Since from the thermodynamic standpoint the only influence that pressure can have on equilibrium in aqueous solution is by changes produced in the partial molal volumes of the constituents ((21) p. 204), in the light of the

This is a factor that modifies the obtained diffusible calcium from the true diffusible calcium of the serum. From the many studies carried out on the subject, we know that, with the exception of calcium and magnesium, all other ionic constituents are completely or practically completely diffusible. Then it is only because the completely diffusible constituents, chiefly sodium chloride, are present in relatively such large amounts as compared to the non-diffusible constituents and because of the small equivalent concentration of the proteins of the serum, that values of diffusible calcium obtained by ultrafiltration represent practically the true original diffusible calcium.

TABLE III.
Comparison of Chloride in Serum and Ultrafiltrate.

Experiment No.	Animal.	Chloride in serum.	Chloride in ultrafiltrate.	Ratio of Cl in ultrafiltrate to Cl in serum
		<i>mm per l.</i>	<i>mm per l.</i>	
1	Beef.	103.5	109.0	1.055
2	"	99.5	107.6	1.08
3	Rabbit.	107.4	117.2	1.09
4	Sheep.	103.0	111.3	1.08
5	Beef.	101.9	114.2	1.12
6*	Pig.	106.0	117.0	1.10
7*	"	105.5	117.3	1.115
8*	"	94.8	102.2	1.08

* Data recalculated from Neuhausen and Pincus (4), Table II, p. 102.

Another consideration of importance is the influence of the Donnan equilibrium theory as extended to heterogeneous equilibria by Wilson and Wilson and others (22, 23). According to this development, it is postulated that there is the same spatial arrangement of ionic constituents in the vicinity of a colloidal micelle as there is obtained with a membrane interposed. On this view, ultrafiltration would give in essence the same experimental results

small compressibility of such solutions it is obvious that pressures of even several atmospheres can have very little effect on the values of diffusible calcium. Where differences have been claimed as in the low results obtained by Richter-Quittner (5) with a low filtration pressure, the results are probably due to imperfect filtering membranes.

as a membrane distribution experiment, if the amount of ultrafiltrate fluid removed were too minute to produce appreciable changes due to concentration of the colloidal constituents. No experimental evidence is as yet available to test this idea generally. For blood serum from a comparison of the ultrafiltration results of a completely diffusible constituent such as chloride ion, we can make a test as to whether the ultrafiltration process gives results analogous to a membrane equilibrium distribution. Loeb and Nichols (20) have determined the distribution ratios of chloride and calcium in membrane equilibrium experiments with blood serum. Using a saline solution of about the same concentration as is present in blood for the external solution, they obtained an average ratio of chloride in the external fluid to chloride in the serum of 1.10:1. The chloride in ultrafiltrate and serum has been determined in a few instances by Neuhausen and Pincus (4). To obtain more extensive data, we also have made a number of measurements. The Van Slyke method as modified by Wilson and Ball (24), was used by us in making our chloride determinations. The results are given in Table III. All the analytical results for the chlorides in Table III are given in millimols per liter of serum or aqueous solution. Neuhausen and Pincus' values have been recalculated to this unit. No correction is made for the volume occupied by the proteins. It is to be observed that the chloride ratios of ultrafiltrate to serum approximate the membrane equilibrium ratios obtained by Loeb and Nichols. The presumption is then that ultrafiltration results on blood serum are equivalent to membrane distribution figures.

The alternate interpretation would be that the difference in chloride between ultrafiltrate and serum is due to the specific volume and the water of hydration of the serum proteins. But such an interpretation would apply equally to the membrane distribution experiments of Loeb and Nichols (20). Since this interpretation implies that the Donnan effect between serum and aqueous solution is zero, it is highly improbable.

Equilibrium between Diffusible and Non-Diffusible Serum Calcium.—Updegraff, Greenberg, and Clark obtained essentially identical figures for diffusible calcium when there was employed ultrafiltration alone or combined diffusion and ultrafiltration with the filtering sac immersed in water, isotonic sodium chloride, or

calcium-free Ringer's solution. On the basis of these results they concluded that diffusible and non-diffusible calcium varied independently of each other. The published results since then of Marrack and Thacker (19) and Loeb and Nichols (20) show that such a conception is erroneous. Instead, at least qualitatively, the results show that at constant pH, the amount of non-diffusible calcium is determined by the protein content and the height of the diffusible calcium concentration of the blood stream. The reasons for the contrary results of Updegraff, Greenberg, and Clark at present are difficult to place. Imperfect diffusion between inner and outer liquids or slowness of readjustment of equilibrium with changes of the calcium concentrations are possible explanations. The apparent non-effect of changes in carbon-dioxide tension of the value of diffusible calcium also may perhaps be due to a slowness in readjustment of equilibrium. As has already been pointed out, because of these doubtful points, the combined diffusion and ultrafiltration method has been abandoned by us in favor of pure ultrafiltration.

Since the non-diffusible calcium is a function of the protein and diffusible calcium concentration when the pH is constant, it becomes a problem of considerable interest to determine whether the relation between these factors can be fitted to a mathematical expression. Some of the data of Loeb and Nichols (20) we found suitable for the investigation of such a relationship, and we also found that what provisionally we shall call the "ionized" calcium and "protein-bound" calcium are related to each other according to the Langmuir adsorption isotherm (25). To make the matter clear, it is to be noted, as stated by Loeb and Nichols ((20), p. 688), that the factors governing the diffusibility of the blood serum calcium in a dialysis system are the Donnan membrane equilibrium and the formation of calcium protein complex ions. The mathematical relations governing the Donnan membrane equilibrium are only of concern in the present instance for calculating the ionized and protein-bound calcium. It is the relationship between the calcium of the protein complex and the calculated ionic calcium that we are primarily interested in. From the ratio of chloride in the serum to chloride in the aqueous solution, the amount of calcium in the serum to be expected from the Don-

nan distribution can be calculated if the calcium content of the aqueous solution is known. This follows from the equation

$$(1) \quad [\text{Ca}]_s = r^2[\text{Ca}]_{aq}$$

in which $[\text{Ca}]_s$ and $[\text{Ca}]_{aq}$ are the calcium concentrations of serum and aqueous solutions respectively, and r is the ratio of chloride in aqueous solution to serum. The value of calcium calculated for the serum in this way, we have designated ionic calcium.⁴ The figures obtained in this way subtracted from the total calcium of the serum give the protein-bound calcium. Such calculations have been carried out by Loeb and Nichols. However it must be noted that Loeb and Nichols, in their analysis on serum, use the volume of serum as the standard for their calculations without taking into account the volume occupied by the protein of the serum. Using the value of 0.75 for the specific volume of the serum proteins (26), we have recalculated the results of Loeb and Nichols on the basis of millimols per liter of protein-free solution. This correction tends to increase the value of chloride and calcium in the serum. The ratios of chloride in aqueous solution to chloride in serum become less than that obtained without the correction and the calculated ionic calcium from the Donnan distribution is also less. This necessarily increases the values for protein-bound calcium.

It is found if the reciprocal of the ionic calcium is plotted against the protein concentration divided by the protein-bound calcium a straight line is obtained. When the values uncorrected for serum proteins, as calculated by Loeb and Nichols, are so plotted, not nearly so good a fit is obtained. The reciprocal of the concentration of calcium in the aqueous solution can equally well be used in place of the reciprocal of the ionic calcium of the serum. In both cases straight lines are obtained, but of course with different slopes. A plot of this type yields a straight line when Langmuir's adsorption isotherm is obeyed. We are not implying by this that the protein-bound calcium is adsorbed by the serum protein. It is significant, as Hitchcock (27) has shown,

⁴ We only wish to imply by the term ionic calcium calculated by Equation 1, that it is an approximation to the calcium activity of the serum and therefore mostly ionized in contrast to the protein-bound calcium which presumably is non-ionized.

that for homogeneous systems the Langmuir equation follows from the law of mass action if one constituent in the system is constant.

The recalculated results of Loeb and Nichols that were found suitable for plotting (the first three sets of experiments of Table I, (20) p. 690) are given in Table IV and the plot obtained is shown in Fig. 2. The points of the data fit two lines, Serums 1 and 3 determining the Curve I, and Serum 2, Curve II. This is perhaps to be expected as with altered ratios of albumin to globulin differ-

150-

100

50-

FIG. 2. Illustration of Langmuir's isotherm governing the amount of protein-bound calcium with changes in ionic calcium in serum. Ordinate = $\frac{P}{[Ca]_p}$; abscissa = $\frac{1}{[Ca^{++}]}$. ●, × represent points determining Curve I from Serums 1 and 3, Table IV, respectively. ○ represents points determining Curve II from Serum 2, Table IV; □, Δ, data of Curve I plotted against reciprocal of calcium of aqueous solution.

ences in the values of protein-bound calcium are to be expected. Curve III is the data of Serums 1 and 3 plotted against the reciprocal of the calcium of the aqueous solution. For Curve I, which is fitted by two experimental series and is therefore the most reliable, we have evaluated the constants of the equation.

The equation is

(2)

$$y = 52.6x + 29.4$$

in which $y = \frac{1}{[Ca]_p}$ and $x = \frac{1}{[Ca^{++}]}$ where P represents the protein and $[Ca]_p$ the protein-bound calcium. The units used are gm. per

TABLE IV.

*Recalculated Results of Table I of Loeb and Nichols.**

Effect of Varying Calcium Concentration on Diffusibility of Serum Calcium.

Serum No.	Protein.	Volume occupied by protein.	r corrected.	$[Ca]_s$ calculated, corrected.	$[Ca]_{aq}$	$[Ca^{++}]_s$	$[Ca]_p$	$\frac{1}{[Ca^{++}]_s}$	$\frac{P}{[Ca]_p}$
	gm. per l.	cc. per l.		mm per l.	mm per l.	mm per l.	mm per l.		
1	79	59	1.05	1.615	0.72	0.79	0.825	1.26	96.0
	78	58	1.05	1.68	0.75	0.825	0.855	1.21	91.2
	76	56.5	1.05	2.72	1.36	1.51	1.21	0.653	62.8
	80	59.5	1.045	2.61	1.31	1.48	1.13	0.676	70.8
	79	59	1.05	3.70	1.96	2.16	1.54	0.464	51.3
2	70	52	1.07	1.14	0.43	0.48	0.655	2.07	106.7
	68	50.5	1.07	1.185	0.44	0.495	0.69	2.02	98.6
	70	52	1.06	1.72	0.75	0.84	0.88	1.187	79.7
	70	52	1.07	1.76	0.79	0.89	0.875	1.178	80.1
	67	50	1.05	2.82	1.49	1.67	1.15	0.60	58.3
	70	52	1.07	2.76	1.45	1.625	1.135	0.616	61.7
3	73	54.5	1.06	0.96	0.39	0.44	0.525	2.28	139.3
	70	52	1.06	1.55	0.72	0.81	0.74	1.237	94.4
	70	52	1.065	2.585	1.40	1.57	1.015	0.638	69.0
	68	50.5	1.055	3.52	2.06	2.31	1.21	0.433	56.2
	70	52	1.06	4.36	2.60	2.92	1.44	0.342	48.5
	73	54.5	1.045	4.50	2.59	2.91	1.59	0.344	45.9

* (20) p. 690.

liter for protein and millimols per liter for calcium. In the form of the Langmuir equation, there is obtained

$$(3) \quad \frac{[Ca]_p}{r} = \frac{[Ca^{++}]}{52.6 + 29.4 [Ca^{++}]}$$

Either equation shows that as the ionic calcium is increased indefinitely, the limiting value of the protein-bound calcium approaches 0.34 millimol per gm. of protein per liter. This amounts, in a solution containing 7.6 per cent protein to 2.55

millimols per liter, or 10.2 mg. of protein-bound calcium per 100 cc. The limiting value of protein-bound calcium given by Curve II is even less. This shows that in blood serum the limiting amount of calcium that can be bound by the protein, *i.e.* the non-diffusible calcium, even if the calcium concentration is infinitely increased, is not of much greater magnitude than the non-diffusible calcium already present.

SUMMARY.

1. Improvements in the technique of ultrafiltration including a regulating cylinder that automatically maintains a constant filtering pressure and suitable collodion solutions are described.

2. A system of analysis for total calcium, diffusible calcium, inorganic phosphorus, and serum proteins on the serum from 12 cc. of blood or even less is outlined.

3. It is shown that once the blood clot is formed the time of contact of the serum with the clot has no effect on the serum calcium or its distribution for periods of 24 hours or even longer.

4. The influence of change of pH by changing the CO₂ tension on the distribution between diffusible and non-diffusible calcium has been reinvestigated and, between the range of pH 7 to 8, found to have no influence on the distribution in the time allowed for ultrafiltration.

5. The hypothesis that the same spatial arrangement of ions exists in the neighborhood of a charged colloidal particle as would be obtained by a membrane distribution, in which event ultrafiltration should give results equivalent to a membrane distribution, has been experimentally tested. From analysis of chloride it has been found that practically the same ratios of chloride in filtrate to chloride in serum are obtained by ultrafiltration as by membrane distribution, which favors the spatial arrangement hypothesis.

6. From the data of Loeb and Nichols (20) it has been shown that at constant pH and protein content, the distribution of protein-bound and ionic calcium, with increasing calcium concentration, conforms to the Langmuir adsorption isotherm. From this equation it is seen that the limiting value of protein-bound calcium when the ionic calcium approaches infinity is of the same order as is present in normal blood serum.

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THE CHEMISTRY OF THE LIPOIDS OF TUBERCLE BACILLI.

X. THE SEPARATION OF LIPOID FRACTIONS FROM AVIAN TUBERCLE BACILLI.*

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INTRODUCTION.

The cooperative investigation on tuberculosis sponsored by the Research Committee of the National Tuberculosis Association, in conjunction with various research laboratories, was begun by subjecting a standard human type of *Bacillus tuberculosis*, Strain H-37, to chemical analysis. The several products that were isolated by chemical methods were then submitted to various investigators for a study of biological properties. In view of the fact that the results of these experiments appeared to be of fundamental importance it was decided to include in the research program other acid-fast bacteria. It was hoped that such a comprehensive comparative study would reveal differences in chemical composition that would be of value in interpreting the biological behavior of various closely related organisms.

The avian type of tubercle bacillus was selected as one member of the acid-fast group to be included in this work. The lipid fractions of the avian bacillus have, therefore, been separated by methods as nearly as possible identical with those that were developed in the examination of the lipoids of the human type of bacillus (1). It was quite evident that the avian bacillus yielded a smaller amount of total lipids than the human bacillus and that

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† Holder of a National Tuberculosis Association Fellowship at Yale University, 1928-29.

a larger percentage of lipids was retained in the bacterial residue. It was not practical in this work to attempt to extract the lipids completely because the bacterial residue was valuable and costly and it was wanted for the study of many other constituents besides the fat fractions, particularly sugars, nucleic acid, proteins, etc., and it was believed that the employment of sufficiently drastic methods to remove all fat would seriously alter or decompose the other components. We employed, therefore, only the mildest methods of extraction; namely, alcohol-ether and chloroform at room temperature. Moreover, we were less concerned with absolute values than with such comparative values as would be obtained by applying similar or identical methods of extraction and separation.

A search of the literature has revealed but few publications dealing with the chemical composition of avian tubercle bacilli and so far as we are aware no work comparable to the present investigation has ever been published. The first chemical analysis of avian bacilli that we have found was published by DeSchweinitz and Dorset (2). These authors recorded a total fat content of 30.65 per cent and they also found that the fat contained a notable amount of free fatty acids. Frouin (3), by extracting avian bacilli grown on different media with hot absolute alcohol, obtained from 14 to 40 per cent of alcohol-soluble material, but this author gives no chemical data about the extract. Pfannenstiel (4) found 13.05 per cent of fat in the avian bacilli and also reported that the extract was only slightly acid-fast. Long and Campbell (5) in a study of the lipid content of acid-fast bacteria report 11 per cent of total lipids for avian bacilli. The fat had a saponification number of 194 and it contained 35.7 per cent of unsaponifiable matter. This brief review indicates how little information exists regarding the nature and composition of the lipoids contained in the avian bacilli.

The results of the present investigation indicate that avian bacilli contain phosphatide, wax-like material, and acetone-soluble fat, products essentially similar to those found in the human type. The principal difference lies in the quantitative relations, the avian bacilli yielding much less lipid material.

In order to prevent oxidative changes during the extractions, etc., air was displaced in all of the operations by carbon dioxide

and all solvents were saturated with this gas before being used. Nevertheless, a certain amount of exposure to air was inevitable, especially during filtrations, although carbon dioxide was used as much as possible. The alcohol had been purified by distillation over potassium hydroxide and all other solvents had been freshly distilled.

EXPERIMENTAL.

The bacilli which were used in the present investigation have been described by Dr. William Charles White in a personal communication as follows:

"*Mycobacterium tuberculosis* (avian). Isolated about 1921. Received from Dr. Crawford from the Bureau of Animal Industry. Found to be virulent for chickens when tested in 1927. Hygienic Laboratory No. 531."

The bacilli were grown by H. K. Mulford Company at Glenolden, Pennsylvania, on the Long synthetic medium (6). The cultures, about 2000 1 liter Pyrex bottles each containing 200 cc. of the medium, were planted about April 1, 1928, and incubated for a period of 4 weeks. A heavy growth occurred and the bacteria were harvested on May 2 and 3, 1928.

Collection of Bacteria and Extraction with Alcohol and Ether.

The living bacteria were filtered on large Buchner funnels and washed with water. A stream of carbon dioxide was passed over the funnels during the filtration. Six 20 liter Pyrex bottles were charged with 7 liters of alcohol and 7 liters of ether, the solution was saturated with carbon dioxide, and sufficient moist, washed bacteria were then added to fill the bottles. The contents were shaken until a homogeneous mixture resulted. The bottles were securely stoppered and shipped to the Sterling Chemistry Laboratory at New Haven where they were kept in a cold room until August 17, 1928. The material was then brought into the laboratory and shaken occasionally for 3 days. The bacteria settled rapidly, leaving a clear, slightly amber-colored, supernatant fluid.

The clear extract was syphoned off under carbon dioxide pressure and the bacterial residue was filtered on Buchner funnels and washed with a 50 per cent alcohol-ether solution. The filtrate and

washings were united with the main extract. The bacterial residue was extracted with chloroform at room temperature for 6 weeks as will be described later.

Alcohol-Ether-Soluble Lipoids.

The clear alcohol-ether extract was concentrated at a low temperature, an atmosphere of carbon dioxide being maintained by bubbling a stream of the gas through the solution. The ether was distilled off at atmospheric pressure and the alcohol was removed under reduced pressure.

The aqueous suspension of fats that remained was extracted four times with large quantities of ether. Persistent emulsions formed which broke very slowly. The emulsions deposited a small amount of solid wax-like particles which were collected and later added to the chloroform-soluble wax. The aqueous solution was finally extracted twice with chloroform. On evaporation of the chloroform extract only 1.2 gm. of fatty residue were obtained which were combined with the ethereal solution.

The fat-free aqueous solution was examined for water-soluble sugars as will be described later.

The ethereal solution, which contained all of the alcohol-ether-soluble lipoids, was concentrated to a volume of about 700 cc. It was brown in color and towards the end of the concentration a portion of the lipoids separated as a syrupy layer on the bottom of the flask. The mixture was shaken and diluted with an equal volume of gold acetone, when a precipitate formed which was partly gummy and partly in a solid granular condition. On standing overnight at room temperature the precipitate, which consisted of crude phosphatide, collected as a gummy mass on the bottom of the flask. The dark brown supernatant solution was decanted off and the precipitate was washed with acetone, the washings being added to the original mother liquor.

The ether-acetone solution was saved for the separation of the acetone-soluble fat.

Purification of the Phosphatide.

The crude precipitate, obtained as mentioned above, was dissolved in 350 cc. of ether and the slightly cloudy solution was precipitated by adding 700 cc. of acetone. The solid granular pre-

cipitate was filtered and washed with acetone. The material was precipitated a second time in the same manner and it was then dissolved in 250 cc. of ether. The solution was transferred to a tall cylinder, the slight amount of insoluble matter was allowed to settle, and the nearly clear solution was decanted. The insoluble matter was later combined with the wax fraction.

The ethereal solution of the phosphatide was precipitated by adding 500 cc. of acetone and the precipitate was filtered off and washed with acetone.

For further purification the substance was precipitated five times from 200 cc. of ether by adding 200 cc. of acetone. The material separated each time as a gummy mass that adhered to the sides of the flask. The supernatant fluids were decanted and the precipitate was rinsed with acetone.

For the final separation the substance was dissolved in 200 cc. of ether and the solution was poured with constant stirring into 700 cc. of ice-cold acetone. When the phosphatide is precipitated in this manner, it is obtained as a coarsely granular, nearly white, powder. The substance was filtered off, washed with acetone, and dried *in vacuo* over sulfuric acid.

The dried phosphatide weighed 71.8 gm. In general properties the substance resembled closely the phosphatide, Fraction A-3, obtained from the human type of tubercle bacillus.

For analysis the substance was dried at 56° *in vacuo* over dehydrite. The loss in weight was 1.33 per cent.

Analysis.

0.4934 gm. substance: 0.0387 gm. $Mg_2P_2O_7$.

0.4934 " " : (Kjeldahl) 1.70 cc. 0.1 N HCl.

0.5109 " " : 0.0476 gm. ash.

Found. P 2.18, N 0.48, ash 9.31.

The phosphatide was submitted to Dr. Sabin for physiological experiments and it was found to possess a biological activity which was very similar to that of the phosphatide, Fraction A-3, from the human bacillus.

The ether-acetone mother liquors, obtained in the purification of the phosphatide, were combined and concentrated to dryness under reduced pressure. The residue was dissolved in ether and precipitated by adding acetone. The substance was again dis-

solved in ether and the solution was poured into cold acetone, when a nearly white granular precipitate separated. After the precipitate had been filtered, washed with acetone, and dried it weighed 7.9 gm. and in properties it closely resembled the first fraction of the phosphatide. The total yield of phosphatide from the avian bacillus was, therefore, 79.7 gm.

The remaining mother liquors, after the separation of the second phosphatide fraction, were concentrated under reduced pressure, and the residue after it had been dried weighed 20 gm. It formed a brown soft solid which probably consisted largely of acetone-soluble fat and will be designated acetone-soluble fat, Fraction I.

Separation of Acetone-Soluble Fat.

The original dark brown ether-acetone mother liquor was concentrated to about 1 liter and the solution was cooled in ice water. A considerable amount of a solid precipitate separated which was filtered off and washed with acetone.

The precipitate was only slightly soluble in ether, benzene, toluene, or acetone, but it was readily soluble in chloroform. It was, therefore, dissolved in chloroform and precipitated by the addition of methyl alcohol. The substance weighed 16.1 gm. and since its properties resembled those of crude tubercle bacillus wax it was later combined with the chloroform-soluble wax.

The mother liquors, after removal of the wax fraction mentioned above, were concentrated under reduced pressure and the residue was dried in a vacuum desiccator. It formed a dark brown soft solid which weighed 57.3 gm. This substance represents the acetone-soluble fat, Fraction II.

Extraction of Bacillary Residue with Chloroform.

The filtered and washed bacterial residue, after extraction with alcohol and ether, was distributed between two 20 liter Pyrex bottles and sufficient chloroform was added to fill the bottles. The mixture was allowed to stand at room temperature with occasional stirring for 6 weeks. The bacteria, which did not settle in the chloroform extract but formed a perfect suspension, were filtered on Buchner funnels, with hardened filter papers. The filtration was very slow. Since the bacterial mass could not be washed on the funnels, it was returned to a 20 liter bottle,

shaken with chloroform, and again filtered as before. A sample of the bacteria was extracted a third time with chloroform but the extract contained very little solid residue on evaporation. The bacterial residue was, therefore, dried in a vacuum oven at 37°. It formed a leathery mass which was difficult to dry and very difficult to pulverize. The dry mass which was finally obtained weighed 2927.7 gm. An appreciable quantity of lipid material was still retained within the bacterial cells, which could be liberated by heating with acids. But since such a treatment would largely destroy other valuable constituents, it was decided to reserve the bacterial residue in its present condition for future work on proteins, carbohydrates, etc.

Examination of Chloroform Extract.

Fractions of wax-like material that had been separated from the alcohol-ether-soluble lipids were dissolved in the chloroform extract. Owing to the presence of a small amount of suspended bacteria, the chloroform extract was cloudy. It was filtered under carbon dioxide pressure through a Chamberland candle and the candle was washed several times with chloroform.

The bacterial residue, after it had been removed from the candle and dried, weighed 15 gm. It was added to the main lot, thus giving a total of 2942.7 gm. of dry extracted bacteria.

The candled chloroform extract, a brilliantly clear, light yellow solution, was concentrated under reduced pressure to a volume of about 1200 cc., when some oily material began to separate. The solution was cooled and mixed with 1 liter of ice-cold methyl alcohol which caused a heavy, light yellowish precipitate to separate. The material was filtered off, washed with methyl alcohol, and dried. The substance, which corresponds in properties to crude tubercle bacillus wax, weighed 321 gm.

The mother liquor on evaporation to dryness yielded 58.5 gm. of a more highly colored waxy residue. The total chloroform-soluble wax-like material obtained from the avian bacilli amounted, therefore, to 379.5 gm.

Isolation of Polysaccharide from Aqueous Solution.

The aqueous alcohol solution from which the ether-soluble lipids had been removed measured about 15 liters. On account

of excessive foaming this solution could not be concentrated by distillation. It was, therefore, precipitated with a slight excess of lead acetate and the heavy white precipitate was filtered off and washed with water. The lead salt was suspended in water and decomposed with hydrogen sulfide. The lead sulfide was filtered off and the filtrate was concentrated to a thick syrup under reduced pressure and the material was reserved for examination.

The clear yellow filtrate from the lead acetate precipitate was concentrated to a volume of 1 liter under reduced pressure with the occasional addition of a few drops of amyl alcohol to control the foaming. A slight precipitate which had separated was removed and an excess of basic lead acetate was added to the clear

TABLE I.

Lipoid and Sugar Fractions Separated from About 2000 Cultures of Avian and Human Tubercle Bacilli.

	Avian bacilli.	Human bacilli.
	gm.	gm.
Phosphatide, Fractions 1 and 2.....	79.7	253.1
Acetone-soluble fat, Fractions I and II.....	77.3	240.0
Chloroform-soluble wax, Fractions 1 and 2.....	379.5	427.0
Polysaccharide.....	36.1	33.9
Dried bacterial residue.....	2942.7	2902.0
Total dry bacillary material.....	3515.3	3868.5
“ lipoids.....	536.5	920.1

solution. A slight precipitate separated and was removed. The filtrate was made strongly alkaline with ammonium hydroxide and the mixture was allowed to stand overnight. The precipitate was collected by centrifuging, washed with water in the centrifuge cups, finally filtered on a Buchner funnel, and washed with water. The washed precipitate was decomposed with hydrogen sulfide in the usual manner and the resulting solution was concentrated to a thick syrup under reduced pressure. The syrup was dried in a vacuum desiccator and finally ground in a mortar under absolute alcohol until a grayish powder resulted. The solid substance was filtered, washed with alcohol, and dried *in vacuo*. The alcoholic solution was again concentrated to a thick syrup and the above treatment was repeated, when a smaller quantity of a powder was

obtained which was combined with the first lot. The dried material weighed 36.1 gm. and it is analogous to the polysaccharide which was isolated in a similar manner from the human type of tubercle bacillus. The substance is very soluble in water and it gives no direct reduction with Fehling's solution but after boiling for several minutes with hydrochloric acid it reduces Fehling's solution.

A summary of the lipoids obtained from the avian tubercle bacillus is given in Table I and for comparison we include the quantities obtained under an identical procedure from the human bacillus. The values found indicate a striking quantitative difference in the various constituents. The amount of phosphatide and acetone-soluble fat obtained from the avian is only a small fraction of that produced by the human tubercle bacillus.

The phosphatide is the only one of these products that we have had time to analyze, but we hope to determine the composition of the other fractions in the near future.

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THE CHEMISTRY OF THE LIPOIDS OF TUBERCLE BACILLI.

XI. THE PHOSPHATIDE FRACTION OF THE AVIAN TUBERCLE BACILLI.*

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INTRODUCTION.

In Paper X of this series (1) we described the separation of a phosphatide from the alcohol-ether-soluble lipoids of the avian tubercle bacillus and indicated briefly that the preparation in chemical and physiological properties closely resembled the phosphatide, Fraction A-3, which had been isolated previously from the human tubercle bacillus (2). In order to compare the composition of the two products we have determined the various cleavage products which are formed when the avian phosphatide is hydrolyzed.

The investigation has shown in general that the phosphatides from the avian and the human bacilli yield similar cleavage products. The preparation from the avian bacillus contains, however, a smaller percentage of ether-soluble and a higher percentage of water-soluble constituents. Both preparations contain practically the same percentage of phosphorus and nitrogen.

The ether-soluble constituents consisted mainly of fatty acids. A small amount of wax-like material was also present which was probably due to a slight contamination of the phosphatide with wax. The fatty acids were composed of solid saturated acids, presumably a eutectic mixture of palmitic and stearic acids, and

* The present report is a part of a cooperative investigation on tuberculosis and it has been supported partly by funds provided by the Research Committee of the National Tuberculosis Association.

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liquid fatty acids. The liquid fatty acids contained nearly equal parts of oleic acid and a liquid saturated fatty acid of high molecular weight which was analogous to phthioic acid (3) although it differed from other specimens of crude phthioic acid obtained from the human bacilli in that it was optically inactive. When this liquid saturated fatty acid was tested in Dr. Sabin's laboratory at the Rockefeller Institute, it was found to possess a biological activity similar to that of tuberculostearic acid.

The avian phosphatide yielded, after hydrolysis, nearly 50 per cent of water-soluble constituents, among which we identified glycerophosphoric acid, free phosphoric acid, some free glycerol, and glucose. In addition, the solution contained a substance which is probably a carbohydrate acid similar to the acid obtained from the phosphatide, Fraction A-3 (2), but this acid has not yet been identified. The aqueous solution also contains practically all of the nitrogen which was present in the phosphatide. Up to the present time we have been unable to isolate the nitrogen-containing compound in solid form. It is soluble in water and in alcohol but it gives no alcohol-insoluble compound with platinic chloride, hence it cannot be choline.

We have retained the name phosphatide to designate the phosphorus-containing lipoids obtained from tubercle bacilli although these products differ greatly in composition from the usual phosphatides. The phospholipids from tubercle bacilli contain about 2.2 per cent of phosphorus and only about 0.4 per cent of nitrogen. In addition to fatty acids and glycerophosphoric acid these compounds yield on hydrolysis large amounts of water-soluble carbohydrates. The reducing sugar that is formed is evidently glucose because it gives an osazone which possesses the properties of glucosazone, but in addition to the reducing sugar we obtained about an equal amount of an organic acid, apparently a sugar acid, which has not yet been identified.

The carbohydrate complex is probably in chemical combination in the phosphatide molecule. An aqueous suspension of the original phosphatide gives no evidence of reducing sugars when tested with Fehling's solution, but after the substance has been completely hydrolyzed with mineral acids a heavy reduction of Fehling's solution is obtained.

The results of the physiological experiments with the avian

phosphatide and with the liquid saturated fatty acid have been summarized as follows by Dr. F. R. Sabin and Dr. C. A. Doan of the Rockefeller Institute.

"The Avian Phosphatide.—In preliminary tests of the avian phosphatide the tissue reactions in two rabbits were similar in kind to those observed after the human and bovine phosphatides, but considerably less in magnitude. The lesions were in part in the form of tubercle-like bodies with extensive infiltration of lymphocytes, but more in the form of diffuse epithelioid cells and Langhans' giant cells. There were more leucocytes than are usually found after twelve intraperitoneal injections of bovine or human phosphatide, and an occasional small abscess. This either indicates a more irritating reaction of the avian phosphatide for rabbit tissues or the presence of a secondary infection in the two animals studied. Cultures were negative for pyogenic organisms in each instance, but further biological studies are necessary to fully establish any points of difference in the reactions to the three phosphatides of different origin.

Avian Fatty Acid.—Twelve intraperitoneal injections, each of 20 mg. of avian fatty acid in 0.5 cc. mineral oil, produced no typical tubercular tissue. There were a few scattered epithelioid cells, an occasional epithelioid giant cell and many foreign body giant cells. In general the reaction was similar to that following the optically inactive tuberculostearic acid from the human, H-37 strain, and not like that of the fatty acid derived from the human phosphatide.

The Avian Phosphatide As Antigen.—When given intravenously in small antigenic doses the avian phosphatide produces in rabbits' serum a precipitating substance which may be used to test for the presence of this antigen in avian tuberculosis. Preliminary studies indicate that there are at least quantitative differences in the specificity of the phosphatides from human, avian and bovine tubercle bacilli, and that they may be used to assist in the identification of the type of organism causing any particular case of tuberculosis."

EXPERIMENTAL.

The phosphatide fraction had been prepared and purified as described in a former paper (1). The freshly isolated moist preparation was readily soluble in ether, chloroform, and benzene, but after the substance had been dried, the solubility in ether decreased. After the phosphatide had stood for some time in a bottle under carbon dioxide it did not dissolve completely in ether, but it was still readily soluble in chloroform and in benzene. The substance was a nearly white, coarsely granular powder. Heated in a capillary tube, it began to darken at 190°, melted at 210–215°, and decomposed at 220°, forming a dark brown liquid. On moist

litmus paper it showed a strongly acid reaction. When the substance was rubbed up with water, it formed an opalescent colloidal solution which when sufficiently diluted with water became practically clear.

When the colloidal solution was boiled with Fehling's solution, it became perfectly clear and showed no reduction. The colloidal solution was very stable and it was only partly coagulated by the addition of acids, alkalies, or salts; the acidified solution formed a coagulum when it was heated to boiling, while the alkaline solution became perfectly clear on heating. The avian phosphatide was more stable than the phosphatide, Fraction A-3, and required several hours longer boiling with dilute sulfuric acid before it was completely hydrolyzed.

For analysis the substance was dried at 56° *in vacuo* over dehydrite. The loss in weight was 1.33 per cent.

0.4934 gm. substance: 0.0387 gm. $Mg_2P_2O_7$.

0.4934 " " : (Kjeldahl) 1.70 cc. 0.1 N HCl.

0.5109 " " on combustion left 0.0476 gm. ash.

Found. P. 2.18, N 0.48, ash 9.31.

Hydrolysis.

The usual precautions were taken for the displacement of air by carbon dioxide during the hydrolysis and until the unsaturated acid had been reduced. The phosphatide, 14.6510 gm., was rubbed to a suspension in 400 cc. of water, 10 cc. of concentrated sulfuric acid were added, and the mixture was refluxed for 16 hours. The coagulum which formed at first was gradually changed into an oil which solidified on cooling. The fatty acids were extracted with ether and the ethereal solution was washed with water, the washings being added to the main aqueous solution which was reserved for the determination of water-soluble constituents.

The ethereal solutions formed rather persistent emulsions which broke slowly on standing and at the same time a small amount of solid particles separated which was filtered off, washed with ether, and dried. This material, which was of wax-like consistency, weighed 0.27 gm.

The washed and filtered ethereal solution was concentrated and the residue was dried in a vacuum desiccator. The semicrystal-

line soft solid weighed 8.6 gm. The material was treated with 50 cc. of warm alcohol, but as it did not dissolve completely the solution was made alkaline with potassium hydroxide, diluted with water, and extracted with ether. The ether extract was washed with water and the ether was distilled, when 0.5 gm. of a waxy residue was obtained.

The alkaline solution, after the ether extraction, was acidified and extracted with ether. The ethereal solution was washed with water, the ether was distilled, and the fatty acids were dissolved in 50 cc. of alcohol, giving a clear solution. After being neutralized with potassium hydroxide, the solution was diluted with water, precipitated with an excess of lead acetate, and the lead soaps were filtered off, washed with water, and dried *in vacuo*. The dried lead soaps were treated with ether and the ether-insoluble and ether-soluble lead salts were decomposed by shaking with dilute hydrochloric acid. The fatty acids were isolated in the usual manner and dried *in vacuo*. The solid saturated fatty acids weighed 2.7 gm. and the liquid fatty acids weighed 5.1 gm.

Saturated Fatty Acids.

The saturated solid fatty acids were dissolved in alcohol, treated with norit, filtered, and the filtrate was cooled in ice water. The white crystalline substance that separated was recrystallized, three times from methyl alcohol and twice from acetone, and was obtained as snow-white crystalline aggregates. The acid melted at 56–57°, solidified at 52°, and remelted at 55–56°.

A second fraction was isolated from the mother liquor, which melted at 56–57°, solidified at 53°, and remelted at 56–57°.

Titration.—0.4009 gm. of acid dissolved in 50 cc. of neutral alcohol, with phenolphthalein as indicator, required 15.09 cc. of 0.1 N alcoholic KOH. Found, molecular weight 265.

The acid probably represents a eutectic mixture of palmitic and stearic acids.

Liquid Fatty Acids.

The iodine number of the liquid fatty acids, as determined by the Hanus method, was 36.84. The low iodine number indicated the presence of a large percentage of a liquid saturated fatty acid. In order to remove the unsaturated fatty acid the mixture of liquid

acids was reduced with hydrogen and platinum oxide (4) and the reduction product was separated into solid and liquid fatty acids by means of the lead soap-ether treatment in the same manner as described under phthioic acid (3).

Reduced Acid.

The acid isolated from the ether-insoluble lead soaps was a white crystalline solid which weighed 2.7 gm. The substance was crystallized from alcohol and recrystallized twice from acetone. The snow-white crystals melted at 68°. After two further crystallizations from acetone the melting point was 69–70° and it did not change on recrystallization from methyl alcohol and from acetone. A mixed melting point with pure stearic acid showed no depression.

Titration.—0.3292 and 0.5185 gm. of acid dissolved in 50 cc. of neutral alcohol, with phenolphthalein as indicator, required 11.45 and 18.15 cc. of 0.1 N alcoholic KOH. Found, molecular weight 287, 285. Calculated for stearic acid, $C_{18}H_{36}O_2$, molecular weight 284.

The results given above indicate that the top fraction of the solid saturated fatty acid which was isolated after reduction was stearic acid and it is probable that oleic acid was the only unsaturated acid present in the original liquid acids. It must be mentioned, however, that a less pure fraction of the reduced acid which melted at 64–66° was obtained from the mother liquors.

The amount of the solid saturated fatty acid, 2.7 gm., which was isolated from the ether-insoluble lead salts after reduction, was somewhat larger than the value calculated from the iodine number of the crude liquid acids. The discrepancy must be due to the incomplete removal of the solid saturated fatty acid in the first lead soap-ether treatment.

Liquid Saturated Fatty Acid.

The fatty acid obtained from the ether-soluble lead soap, after the catalytic reduction of the unsaturated acid, was a light yellow oil which weighed 2.07 gm. The acid contained a slight amount of some substance which was not soluble in cold alcohol. The oil was therefore mixed with alcohol, treated with norit, and filtered. The norit was then extracted with chloroform and the extract was

evaporated to dryness, when 0.1 gm. of a wax-like residue was obtained. The clear alcoholic solution of the acid was neutralized with potassium hydroxide, diluted with water, and precipitated by adding an excess of lead acetate. The lead soap was washed with water, treated with ether, and the solution, which was faintly cloudy, was filtered. The clear ethereal solution was shaken with dilute hydrochloric acid, washed with water, and the fatty acid was isolated in the usual manner. After the substance had been dried, it weighed 1.8 gm. and it was a light yellow oil. It solidified when cooled in ice water and liquified below 15°. It was miscible in all proportions with alcohol. The acid was saturated since in chloroform solution it did not decolorize bromine. It was optically inactive; 0.7523 gm. of acid dissolved in alcohol and made up to 10 cc. showed no rotation in a 1 dm. tube.

Titration.—0.5620 gm. of acid dissolved in 50 cc. of neutral alcohol, with phenolphthalein as indicator, required 18.53 cc. of 0.1 N alcoholic KOH. Found, molecular weight 303.

The liquid saturated fatty acid, described above, corresponds in general properties to the crude phthioic acid (3) which was obtained from the phosphatide, Fraction A-3, but it differs from phthioic acid in that it is optically inactive and when the acid was tested in Dr. Sabin's laboratory at the Rockefeller Institute it was found to give a somewhat different reaction from that of phthioic acid.

Examination of Water-Soluble Constituents after Hydrolysis of Phosphatide.

(a) *Determination of Reducing Sugar.*—The aqueous solution and washings, after extraction of the fatty acids with ether, were concentrated under reduced pressure and made up to a volume of 250 cc. Reducing sugars were determined in 5 and 10 cc. portions of the solution by Fehling's solution. Found, cuprous oxide 0.1055 gm. and 0.2162 gm., corresponding to 16.3 and 16.8 per cent of glucose.

(b) *Determination of Solids.*—The remaining portion of the solution was freed quantitatively of sulfuric acid by barium hydroxide, filtered, concentrated, and again made up to 250 cc. 10 cc. of the solution were dried to constant weight in a vacuum desiccator. The residue weighed 0.2452 gm. which corresponds to 47.56 per cent of solids in the original solution.

(c) *Precipitation of Sugar Acid with Phenylhydrazine*.—To 50 cc. of the solution, representing 2.7542 gm. of the phosphatide, were added 2 gm. of phenylhydrazine dissolved in a few cc. of alcohol. A nearly colorless crystalline precipitate began to separate and after the mixture had stood for some time the precipitate was filtered off, washed with water, and dried *in vacuo*. The substance weighed 0.55 gm., corresponding to 19.96 per cent of the phosphatide. It melted with decomposition at 192°. A similar compound was isolated from the water-soluble cleavage products from the phosphatide, Fraction A-3 (2), and it represents apparently a phenylhydrazine salt of a sugar acid. Lack of time has prevented any examination of this substance.

(d) *Preparation of Glucosazone*.—After filtering off the precipitate as mentioned under (c), the excess of phenylhydrazine was removed by extraction with ether and the solution was concentrated to 20 cc. The solution was heated on the water bath with phenylhydrazine hydrochloride and sodium acetate. The osazone that separated was purified by several recrystallizations from dilute alcohol. The yellow, needle-shaped, crystals melted with decomposition at 207–208°. The properties agree with those of glucosazone.

(e) *Isolation of Barium Glycerophosphate*.—The balance of the solution, after (b) and (c), was made slightly alkaline by adding barium hydroxide, and 150 cc. of alcohol were added. The voluminous white precipitate was filtered off, after it had stood overnight, washed with alcohol, and dried *in vacuo*. The substance which weighed 2.2154 gm. was treated with 25 cc. of cold water. The insoluble portion was filtered off, washed with a little water, and dried. It weighed 0.8265 gm., and, as it consisted principally of barium phosphate, it was discarded. The barium glycerophosphate which was contained in the filtrate was precipitated by adding alcohol and after it had been filtered, washed, and dried it weighed 1.2387 gm. It was purified by several precipitations from water by alcohol and was obtained as a snow-white powder. For analysis it was dried at 105° *in vacuo* over dehydrite.

0.1915 gm. dried substance: 0.1274 gm. BaSO₄ and 0.0660 gm. Mg₃P₂O₇.

C₃H₇O₄PBa·2H₂O. Calculated. Ba 40.01, P 9.02.

Found. " 39.14, " 9.60.

The substance had the properties of barium glycerophosphate but the values found on analysis do not agree with calculated composition of this substance, the percentage of barium being much too low.

(f) *Examination for Choline*.—The dilute alcoholic solution which remained, after removal of the barium phosphate and glycerophosphate, was freed quantitatively of barium by sulfuric acid and filtered. The filtrate was neutralized with hydrochloric acid and concentrated under reduced pressure to dryness, the last moisture being removed by evaporation several times with ab-

TABLE I.
Principal Cleavage Products of Avian Phosphatide.

	Analysis 1.	Analysis 2.
	<i>per cent</i>	<i>per cent</i>
Wax-like material.....	5.93	8.36
Mixture of palmitic and stearic acids.....	18.42	16.72
Oleic acid after reduction to stearic acid.....	18.42	17.73
Liquid saturated fatty acid analogous to phthioic acid.....	14.12	16.72
Glucose..	16.50	14.70
Sugar acid obtained as salt with phenylhydrazine.	19.96	
Glycerophosphoric acid..	6.07	
Magnesium..		0.52
Potassium.....		0.83
Sodium.....		0.68

solute alcohol. The residue was extracted with alcohol, and the extract was filtered and mixed with an alcoholic solution of platinum chloride. Since no precipitate occurred, we may conclude that choline was absent.

(g) *Examination for Glycerol*.—The solution, mentioned above, was freed from platinum and it was again concentrated to dryness. The residue was extracted with alcohol and the alcohol was evaporated, leaving a thick syrupy residue. When some of the syrup was heated with acid potassium sulfate, the odor of acrolein was noticed, thus indicating the probable presence of some free glycerol.

(h) *Isolation of Crystalline Substance*.—The residue, from (f),

which was insoluble in alcohol was dissolved in a little water and on standing colorless crystals separated. After the crystals had been filtered off, washed with alcohol, and dried, they weighed 0.27 gm. Various portions of a similarly crystalline product were obtained not only from the avian phosphatide but also from the phosphatide, Fraction A-3. The dried crystals melted with decomposition at about 215°. The substance is probably an organic acid but we have not yet had time to examine it thoroughly.

(i) *Nitrogen-Containing Compound*.—Our observations on this subject may be summarized as follows: The compound is soluble in water and in alcohol, but it does not give an alcohol-insoluble platinum double salt, hence choline is not present. Precipitation with phosphotungstic acid has not led to any tangible results. In the Van Slyke procedure about one-half of the nitrogen is liberated. The original solution contains only a small amount of ammonia.

A second analysis was made of the avian phosphatide, with about 20 gm. of material, and the results are summarized in Table I. The values obtained in the two analyses are essentially similar.

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THE CHEMISTRY OF THE LIPOIDS OF TUBERCLE BACILLI.

XII. THE SEPARATION OF THE LIPOID FRACTIONS FROM BOVINE TUBERCLE BACILLI.*

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INTRODUCTION.

In accordance with a general plan for the cooperative investigation on tuberculosis, which is sponsored by the Research Committee of the National Tuberculosis Association, a series of comparative studies of the chemical and biological properties of various types of tubercle bacilli and related acid-fast organisms have been initiated. The bovine type of tubercle bacillus has been included in this program and the present report deals with the separation of lipid fractions from the bovine bacillus.

The literature contains very little definite information regarding the lipoids of the bovine bacillus, but such data as we have been able to find may be summarized as follows: DeSchweinitz and Dorset (1) obtained a total of 26.32 per cent of fat by extraction with ether, alcohol, and chloroform. The fat was found to contain some free fatty acids. Frouin (2) reported that the fat content of different strains of the bacillus varied from 8.55 to 12.99 per cent when grown on media free from glycerol while when glycerol was present the fat varied from 22.95 to 45.51 per cent. Long and Campbell (3) found that the bovine bacillus, Strain B, grown on a synthetic media containing glycerol yielded on successive extraction with hot absolute alcohol and with petroleum ether a total of 22.3 per cent of fat. The fat had a saponification number of 139 and it contained 60 per cent of unsaponifiable matter. Terroine and Lobstein (4) in experiments with

* The present report is a part of a cooperative investigation on tuberculosis and it has been supported partly by funds provided by the Research Committee of the National Tuberculosis Association.

† Holder of a National Tuberculosis Association Fellowship at Yale University, 1928-29.

different types of tubercle bacilli found that the bovine type gave 25.52 per cent of total fat which after saponification contained 67 per cent of fatty acids and 23 per cent of unsaponifiable matter. The fatty acids had an iodine number of 14.3 and melted at 47.5°. These authors also reported that the unsaponifiable matter contained cholesterol corresponding to 1.8 per cent of the fat. Goris (5) in his work employed a mixture of human and bovine bacilli. The use of such a mixture makes it, unfortunately, impossible to evaluate his results with respect to either organism.

Since no definite information has been published regarding the nature of the chemical compounds occurring in the lipoids obtained from the bovine bacillus, we found it necessary to start with first principles and we have attempted to fractionally separate the lipid constituents by methods analogous to those developed in this laboratory for similar investigations on the human (6) and avian (7) bacilli. The results obtained in this work indicate that the bovine bacillus on extraction with alcohol and ether yields principally phosphatide and acetone-soluble fat together with a small quantity of wax-like material while the subsequent extraction of the bacteria with chloroform yields mainly wax. These lipid fractions are essentially similar to those previously isolated from the human and avian bacilli, but in comparing the amounts of the various fractions obtained wide variations between the different types of the three organisms are evident. Moreover, certain distinct differences in properties and in composition of the phosphatide fraction places this product in an intermediate position between the corresponding fractions obtained from the human and avian bacilli. The apparent difference in chemical composition of the phosphatide is also reflected in its biological properties. According to preliminary results observed in Dr. Sabin's laboratory, the biological reaction of the bovine phosphatide is similar to but not identical in intensity with that of the human and avian phosphatides.

The methods of extraction that we employed in this investigation yielded only 13.4 per cent of total lipoids. The bacterial residue still contained a large amount of fat or wax-like material which could only be removed after drastic treatment with alkali or acids. No attempt was made to remove the total lipoids since in order to do so other important bacterial constituents such as sugars, proteins, etc., would have been destroyed.

Lack of time has prevented a complete chemical examination

of any of the various lipid fractions that have been isolated, but work on these substances is under way and the results will be reported later.

EXPERIMENTAL.

The bacteria which were used in this investigation have been described as follows by Dr. William Charles White in a personal communication:

"Mycobacterium tuberculosis (bovis). Bovine tubercle bacillus (Dite Vallée). Hygienic Laboratory, No. 523. H. K. Mulford No. 1698. Isolated from a case of pulmonary tuberculosis (bovine) very caseated at end of bronchial ganglion. Isolated by inoculation into guinea pig and recovered on potato glycerine from the pus of the side ganglion. Never varied in virulence. Always kept on potato glycerine at 6 per cent. Regrown each month. Its virulence was tested for rabbits by W. C. White, Hygienic Laboratory, 12/6/27. 1 mgm. moist weight in ear vein of rabbit, 2500 to 2900 grams. Kills with fine miliary tuberculosis of lungs and kidneys in from 25 to 44 days. Received from Dr. White, April 7, 1927. Adopted as bovine type tubercle bacillus."

The bacteria were grown on the Long synthetic medium (8) in the laboratories of H. K. Mulford Company at Glenolden, Pennsylvania, and the usual 2000 cultures were provided for our work. The cultures were planted from September 14 to 28, 1928, and they were harvested on January 9 to 11, 1929. The moist washed bacteria were evenly distributed between seven 20 liter Pyrex bottles, each bottle containing 14 liters of 50 per cent alcohol and ether. Through an accident one of the bottles was broken and the contents were lost. Consequently about 1700 cultures entered into the investigation but the exact number is not known. The six remaining bottles containing the bacteria were transported by truck to the Sterling Chemistry Laboratory at New Haven.

The usual precautions for displacing the air with carbon dioxide were observed not only in the filtration of the fresh bacteria, but throughout the operations in the laboratory. All solvents used in this work had been freshly distilled and as usual, the alcohol had been distilled over potassium hydroxide.

Extraction of the Bacteria with Alcohol and Ether.

The bacilli were extracted in the alcohol-ether solution at room temperature with occasional shaking for 10 weeks. After shaking,

the bacteria settled rapidly, leaving a clear light amber-colored supernatant fluid. The clear extract was syphoned off under carbon dioxide pressure and the bacterial residue was filtered on Buchner funnels and washed with 50 per cent alcohol and ether and the washings were combined with the main portion of the extract.

The bacterial residue was extracted with chloroform as will be described later.

Separation of Phosphatide from the Alcohol-Ether Extract.

The alcohol-ether extract was concentrated in the usual manner (7), the lipoids were extracted with ether and the ethereal solution was washed with water. No emulsions formed in any of these operations. The ethereal solution was filtered under carbon dioxide pressure through a Chamberland candle when a bare trace of bacterial residue was obtained. The bright clear filtrate was concentrated to 800 cc. when some of the lipoids began to separate as oily drops. On adding 1200 cc. of acetone, a precipitate formed which settled out as a gummy mass on standing. The dark colored supernatant solution was decanted and the precipitated mass was washed with acetone.

The ether-acetone mother liquor and the washings were reserved for the separation of acetone-soluble fat.

The crude phosphatide was precipitated five times from ethereal solution by adding acetone. The mother liquors were either decanted or filtered and the phosphatide was washed with acetone after each precipitation. The phosphatide did not dissolve completely in ether, the solutions containing some finely divided insoluble particles. In order to remove this insoluble material, the ethereal solution was filtered under carbon dioxide pressure through a Chamberland candle and the candle was washed a few times with ether.

A cream-colored solid was scraped off from the candle and after drying *in vacuo* it weighed 10 gm. It was designated Bovine Phosphatide I.

The filtered ethereal solution and washings, which measured 250 cc., were poured into 700 cc. of cold acetone when a somewhat pasty precipitate was obtained which was filtered and washed with acetone. The phosphatide was now precipitated two times by

pouring the ethereal solution into ice-cold methyl alcohol, filtering and washing with methyl alcohol after each precipitation. The substance was obtained as a faintly pink-colored granular powder which after it had been dried *in vacuo* weighed 50.5 gm. and it was designated Bovine Phosphatide II.

The product had now been precipitated eight times from ether, six times with acetone and twice with methyl alcohol. After the ether-insoluble portion, Bovine Phosphatide I, had been removed the substance was easily soluble in ether but after the material had been dried the solubility in ether was very slight. It should also be noted that when an ethereal solution was poured out the film remaining in the flask was very imperfectly soluble in ether. Apparently we are dealing with a substance whose physical properties are very easily altered by drying or by a slight exposure to the air.

Examination of Mother Liquors Obtained in Purifying the Phosphatide.

The ether-acetone mother liquors were concentrated to 300 cc. under reduced pressure, the ether-methyl alcohol mother liquors were then added and the solution was again concentrated to 300 cc. As the ether was removed a precipitate separated and collected as a soft solid on the bottom of the flask. The clear brown supernatant solution was decanted and combined with the first ether-acetone mother liquor which contained the acetone-soluble fat.

The soft solid residue was precipitated three times from ethereal solution by adding methyl alcohol. The last precipitation was done by pouring the cold ether solution into ice-cold methyl alcohol when a yellowish solid granular precipitate was obtained which, after it had been dried *in vacuo*, weighed 114 gm. The substance, which melted below 56°, contained only a small amount of phosphorus and nitrogen and since it appeared to be more like a wax than a phosphatide it was designated Bovine Wax I.

The mother liquors, after separating the waxy substance mentioned above were combined with the ether-acetone solution which contained the acetone-soluble fat.

Separation of the Acetone-Soluble Fat.

The combined ether-acetone and ether-methyl alcohol mother liquors were concentrated under reduced pressure and the residue was mixed with 300 cc. of acetone. The clear dark brown solution was decanted from an insoluble gummy mass. The latter was taken up in ether and the solution was poured into ice-cold methyl alcohol when a wax-like substance precipitated which was filtered, washed with methyl alcohol, and dried *in vacuo*. The dried material weighed 41 gm. and was designated Bovine Wax II.

The acetone solution and the filtrate from the wax mentioned above were combined and evaporated nearly to dryness under reduced pressure. The residue was dissolved in ether, the ethereal solution was dried with sodium sulfate, filtered, and the ether was distilled off. The residue, after it had been dried in a vacuum desiccator, formed a brown soft solid which weighed 128 gm. and it will be designated as acetone-soluble fat.

Preliminary Analyses of the Phosphatide Fractions.

The phosphatide Fractions I and II gave positive tests for phosphorus, nitrogen, and sulfur. After acid hydrolysis the aqueous solution contained reducing sugars. When rubbed up with water both products gave colloidal solutions.

After drying at 56° *in vacuo* over dehydrite the following results were obtained on analysis.

Fraction.	P	N	S	Ash.	Melting point.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
Phosphatide I.....	1 80	1.50			
" II.....	1 87	1 00	0.06	5 33	200-210°
Wax I	0 68	0 34			Below 56°

The bovine phosphatide fractions differ in several particulars, not only in composition but in properties, from the corresponding fractions isolated from the avian and human bacilli. A lower phosphorus and a higher nitrogen content are evident besides traces of sulfur are present. Moreover, Fraction II is extremely difficult to hydrolyze in dilute aqueous acid.

The phosphatide, Fraction II, has been studied by Dr. Sabin

and collaborators at the Rockefeller Institute and the preliminary results have been described as follows in a personal communication from Dr. Sabin:

"Preliminary tests on two rabbits have shown a reaction of specific epithelioid cells and epithelioid or Langhan's giant cells fully as extensive and characteristic as that previously described for the phosphatide from the human strain of tubercle bacilli, H-37. Each animal received 12 intraperitoneal injections of 80 mgm. each of the bovine phosphatide in 10 cc. fresh glass distilled water. In addition to the massive formation of tubercular tissue there were also infiltrations of lymphocytes reproducing the typical appearance of circumscribed tubercles. Upon analysis under higher magnifications the epithelioid cells of the 12th day after bovine phosphatide showed the foamy or even vacuolated cytoplasm seen only during the first 3 days of the human, H-37, phosphatide injections. This we interpret to mean that the bovine phosphatide is dealt with or broken down more slowly by the monocytes of the rabbit than is the phosphatide from the human organism."

In studies conducted by Dr. C. A. Doan (9) of the Rockefeller Institute the phosphatide has been found to give positive precipitin tests with tuberculous serum even in high dilution. The preliminary results are summarized as follows by Dr. Doan:

"The phosphatide, as isolated from the bovine tubercle bacillus, acts as an antigen when introduced intravenously in rabbits, and when tested against the serum of tuberculous cattle is precipitated in high dilutions. These sera are rendered non-reactive after absorption with the homologous phosphatide. While avian and human phosphatides also react with tuberculous cattle sera, the specificity appears to lie in the higher titres found with the bovine phosphatide. Further tests are necessary to establish the diagnostic value of the reaction."

Extraction of Bacterial Residue with Chloroform.

A double extraction of the bacterial residue with chloroform at room temperature was carried out in the manner described under the avian bacilli (7).

The final bacterial residues formed dense tough masses which were difficult to dry and difficult to pulverize. The material was dried in a current of air at room temperature and it weighed 3370 gm.

The bacterial cells still retained a considerable amount of lipoid material which could not be removed by fat solvents without

applying drastic methods of extraction. However, since the material was needed for investigations on proteins, carbohydrates, etc., it was left in its present condition.

Chloroform-Soluble Wax.

The chloroform extract was filtered under carbon dioxide pressure through a Chamberland candle and washed with chloroform. The bacterial cells that were recovered from the candle weighed 20 gm. and were combined with the main portion of the bacterial residue. The clear light yellow filtrate was concentrated under reduced pressure to a volume of 700 cc. The thick brown solution was poured into 1 liter of ice-cold methyl alcohol with vigorous stirring when a yellow granular precipitate separated. The material was filtered off, washed with methyl alcohol, and dried *in vacuo*. It weighed 181 gm. and was designated Bovine Wax III.

The filtrate and washings from above were concentrated under reduced pressure to dryness. The residue, after drying *in vacuo*, weighed only 3.7 gm. It is probably similar to the acetone-soluble fat, and it will be designated acetone-soluble Fat II.

Examination of the Aqueous Portion of the Alcohol-Ether Extract.

The aqueous solution which remained after extracting the ether-soluble lipoids measured about 14 liters. It was filtered through a Berkefeld candle and the filtrate was treated with lead acetate and with basic lead acetate and ammonia in the manner described under the avian bacillus (7). The precipitate produced by basic lead acetate and ammonia was decomposed in aqueous suspension with hydrogen sulfide and the filtrate was concentrated to a syrup. The syrup was ground up in a mortar under absolute alcohol until a powder was produced, the latter was filtered off, washed with absolute alcohol, and dried *in vacuo*. About 43 gm. of a nearly white powder were obtained. The substance corresponds to the polysaccharides obtained from the avian and human bacilli, but the crude product contained some reducing sugars because when dissolved in water it reduced Fehling's solution on boiling. After the substance had been heated with hydrochloric acid the reduction with Fehling's solution was more pronounced. It would seem, therefore, that the substance contains both simple and complex sugars.

The filtrate from the basic lead acetate precipitate was freed from excess of lead with hydrogen sulfide, filtered, and the filtrate was concentrated to a syrup. When the syrup was treated with absolute alcohol, as described above, some 70 gm. of a white

TABLE I.

Alcohol-Ether and Chloroform-Soluble Lipoids from Bovine Tubercle Bacilli.

Fraction.	Weight.	Total dry bacilli.
	gm.	per cent
Phosphatides I and II	60 5	1 53
Acetone-soluble Fats I and II	131 7	3 34
Waxes I, II, and III.	336 0	8 52
Total lipoids	528.2	13 40
Dry bacillary residue	3370 0	85.50
Total dry bacilli	3941.2	

TABLE II.

Lipoids and Other Fractions Separated from Bovine, Avian, and Human Tubercle Bacilli.

Type of organism	Bovine, Strain 523.		Avian, Strain 531.		Human, Strain H-37.	
Approximate No. of cultures	1700		2000		2000	
	gm.	per cent of dry bacteria	gm.	per cent of dry bacteria	gm.	per cent of dry bacteria
Phosphatide, crude.	60 5	1 53	79.7	2 26	253.1	6.54
Acetone-soluble fat	131.7	3 34	77 3	2.19	240 0	6 20
Chloroform-soluble wax	336 0	8.52	379.5	10.79	427.0	11 03
Total lipoids	528.2	13 40	536.5	15.26	920.1	23.78
Polysaccharide	43.0*	1.09	36.1	1.02	33 9	0.87
Dried bacterial residue.	3370 0	85 50	2942 7	83.71	2902.0	75 01
Total dry bacillary material.	3941.2		3515 3		3868.5	

* This figure is included provisionally.

powder were obtained. The aqueous solution of the powder reduced Fehling's solution on boiling but the reduction was more pronounced after heating with hydrochloric acid. It seems evident, therefore, that the water-soluble constituents contain simple as well as complex sugars. Further work will be necessary

before we can separate these constituents and before we can give even an approximate figure representing the polysaccharide fraction.

The lipoids isolated from the bovine bacilli are summarized in Table I.

In Table II is presented a general summary of all the crude products that have been isolated from the three types of tubercle bacilli that have been investigated in this laboratory during the past 3 years.

In conclusion we desire to express our appreciation for the helpful cooperation that has been given by all those who have been interested in various features of this investigation, and particularly to Professor T. B. Johnson, Yale University; Dr. F. R. Sabin and Dr. C. A. Doan, the Rockefeller Institute; Dr. William Charles White, Hygienic Laboratory, and to the H. K. Mulford Company, who at great expense have grown and provided the tubercle bacilli.

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BIOLOGICAL AND CHEMICAL CHANGES IN COW'S OVARIES DURING PREGNANCY.

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Interest in the ovary has been chiefly centered in the lipid fraction because of the fat solubility of the ovarian hormones, at least in their impure condition. In the search for ovarian hormones, chemical examination of the tissues has been largely neglected. The relatively few chemical studies on the ovary at different stages of corpus luteum development have shown but slightly better agreement than have the much more numerous studies based on microscopical staining reactions. The latter, although not adequate for a study of lipid metabolism, at least have the advantage of indicating in what part of the tissues the lipids are located.

Rosenbloom (1) reports no significant changes in the total lipids, phospholipids, neutral fat, fatty acids, cholesterol, and cholesterol esters in the corpus luteum and ovarian residue of the cow during pregnancy. Chauffard, Laroche, and Grigant (2) report an increase in cholesterol content of sow corpus luteum in the later stages of pregnancy. Corner (3) observed a decrease in phosphatides in the corpus luteum of the sow as pregnancy advances. Hermstein (4) has found that at the height of development, human corpora lutea of menstruation contain chiefly cholesterol, cholesterol esters, and phosphatides. In the retrogressive stages, the complex lipids are less abundant and more fatty acids, neutral fat, and soaps are present. Momigliano (5) has also reported a decrease in phospholipids with an increase in neutral fats during retrogression of human corpora lutea.

The work here reported was outlined as a parallel study of the biological activity and chemical composition of the ovary at different stages of pregnancy with the purpose of possibly correlating chemical change with function. Ovaries from the cow's pregnant

cycle were chosen for these studies because of the large amount of material available and the comparative ease of accurately estimating the stage of the cycle.

EXPERIMENTAL.

Ovaries from pregnant cows were collected in three fractions representing pregnancies of the following durations: Fraction 1, up to 3 months; Fraction 2, from 3 to 6 months; Fraction 3, from

TABLE I.
Average Weights and Moisture Content of Ovaries.

Gland fraction.	Average wet weight per gland.	Average dry weight per gland.	Moisture.
	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>
Blank.*			
1	5.25	0.85	84
2	4.77	0.92	81
3	3.69	0.76	80
Ovarian residue.†			
1	4.41	0.80	82
2	4.59	0.85	82
3	4.68	0.91	81
Corpus luteum.			
1	4.98	1.08	78
2	4.65	1.01	78
3	4.75	1.07	76

* Ovary without corpus luteum.

† Ovary from which corpus luteum has been dissected.

6 to 9 months. Each pair of ovaries is composed of one without a corpus luteum, called a "blank" in this article, and the other containing a corpus luteum. The corpora lutea were carefully hand dissected from the ovarian residue in the corpora lutea-containing ovaries. Thus, the ovaries representing each of the three stages of pregnancy were separated into three fractions, (1) blanks, (2) ovarian residue, and (3) corpora lutea, making a total of nine fractions for analysis. A comparison of the average wet and dry weights of these fractions from 2600 ovaries is given in Table I. The results show a progressive decrease in the size of the blanks as pregnancy progresses.

Determination of Estrus-Producing Hormone.—For this study the fresh glands were used. Immediately after collection, 1000 gm. samples of carefully dissected fractions were ground in a meat chopper and preserved with 2 liters of 95 per cent alcohol. During collection and dissection the glands were continuously chilled with ice.

The aqueous alcohol mixture was decanted and the residue exhausted with hot 95 per cent alcohol. The combined alcoholic extracts were concentrated *in vacuo* in an atmosphere of nitrogen

TABLE II.
Determination of Estrus-Producing Hormone.

Gland fraction.	Unsaponifiable fraction in fresh gland.	Activity of unsaponifiable fraction.	Estrus-producing hormone in fresh gland.
	per cent	mg. per rat unit	rat units per kg.
Blanks.*			
1	0.26	16	162
2	0.25	35	71
3	0.26	60	43
Ovarian residue.†			
1	0.30	48	62
2	0.27	81	33
3	0.27	170	16
Corpus luteum.			
1	0.43	290	15
2	0.43	230	19
3	0.41	100	41

* Ovary without corpus luteum.

† Ovary from which corpus luteum has been dissected.

to a syrup, and the syrup extracted with ether. The ether-soluble fat was saponified by boiling for 1 hour with alcoholic potash in an atmosphere of nitrogen. The unsaponifiable fraction was obtained by ether extraction of the soaps and dissolved in olive oil for injection into spayed rats. The oil solution was injected subcutaneously in two injections at 8 hour intervals. The rat unit is taken as the minimum dose which produces estrus as judged by the smear method in ovariectomized rats weighing 140 ± 20 gm. The results of these tests are summarized in Table II.

Chemical Examination.

For this analysis the dried glands were used. After separation into the previously described fractions, the tissues were dried *in vacuo* below 40° and ground to pass a No. 20 sieve. 200 gm. samples of corpus luteum from each stage of pregnancy were subjected to the following analytical procedure, which is essentially the same as that used by Fullerton and Heyl (6) in their examination of corpus luteum.

The tissue was exhausted with anhydrous ether and the combined extracts concentrated to 200 cc. The residue from ether extraction was exhausted with boiling absolute alcohol and the combined alcoholic extracts concentrated to 250 cc. Aliquots were taken for total solids, total P, and cholesterol determination. Free cholesterol was determined by precipitation as digitonide, and cholesterol present as ester was likewise determined in the filtrate after saponification. The remainder of the ether and alcoholic extracts were emulsified by the method of MacArthur (7) and separated into purified lipid and water-soluble extractive fractions.

Purified Lipid Fractions.—These fractions were made up to volume and aliquots taken for determination of total solids, total P, and total S. Other aliquots were freed from chloroform and hydrolyzed by boiling under a reflux condenser with 0.2 N sulfuric acid for 80 hours. The lipid residues and the acid hydrolysates were separated by the method of Brauns and MacLaughlin (8). The lipid residues were dissolved in ether, made up to 100 cc., and aliquots taken for total nitrogen.

The acid hydrolysates were made up to volume and aliquots taken for determination of total N. Aliquots were analyzed for choline and amino nitrogen according to the procedure of Brauns and MacLaughlin (8). Tests for reducing sugars were negative, thus showing the absence of cerebrosides.

The remainder of the purified lipid fractions was saponified with alcoholic potash and the fatty acids isolated in the usual manner. Iodine values of the mixed fatty acids were determined by the Hanus method.

Water-Soluble Extractives.—This fraction was made up to volume and aliquots taken for total nitrogen determination. A larger aliquot was treated as follows: The solution was made slightly alkaline with barium hydroxide, the ammonia aspirated into 0.02

TABLE III.
Composition of Ether Extracts.

Fraction No.....	Ovarian residue.*			Corpus luteum.		
	1	2	3	1	2	3
Total ether extract.						
Total solids, per cent of dry gland.	3.56	3.77	3.48			
Free cholesterol " " "	0.79	0.79	0.78	0.72	0.49	0.75
Ester " " "	0.28	0.34	0.35	0.97	0.86	0.94
Total P " " "	0.031	0.028	0.029	0.28	0.27	0.27
Purified lipid fraction.						
Total solids, per cent of dry gland...	3.36	3.40	3.33	10.7	11.0	11.0
" N " " purified lipid.	0.52	0.44	0.45	0.61	0.41	0.42
Choline N " " "	0.009	0.007	0.009	0.05	0.07	0.01
Amino N " " "	0.21	0.16	0.17	0.25	0.24	0.33
N in lipid residue " " "	0.14	0.12	0.12	0.07	0.11	0.10
Total P " " "	0.72	0.61	0.66	1.10	0.81	0.70
" S " " "	0.27	0.31	0.43	1.15	0.73	0.89
N:P ratio.	1.60	1.59	1.51	1.22	1.12	1.33
N:(P+S) ratio.....	1.17	1.07	0.93	0.61	0.60	0.60
Iodine No., fatty acids.....	86	79	77	126	128	113
Extractive fraction.						
Total N, per cent of dry gland.	0.002	0.002	0.002	0.008	0.008	0.011

Ovary from which corpus luteum has been dissected.

TABLE
tion of Alc

Fraction No.	Ovarian residue.*			Corpus luteum.		
	1	2	3	1	2	3
Total alcoholic extract.						
Total solids, per cent of dry gland.....	5.75	6.12	6.12	16.9	17.7	18.0
“ P “ “ “	0.104	0.108	0.112	0.34	0.34	0.32
Purified lipid fraction.						
Total solids, per cent of dry gland..	2.60	2.50	2.50	6.77	5.42	5.53
“ N “ “ purified lipid.....	1.88	1.94	1.95	2.12	1.49	1.15
Choline N “ “ “	0.71	0.69	0.78	0.22	0.41	0.15
Amino N “ “ “	0.38	0.41	0.39	0.14	0.43	0.32
N in lipid residue “ “ “	0.43	0.45	0.45	0.33	0.18	0.27
Total P “ “ “	2.92	2.86	2.84	2.30	2.12	1.28
“ S “ “ “	0.31	0.34	0.21	2.65	2.01	1.43
N:P ratio.....	1.42	1.50	1.52	2.04	1.55	1.98
N:(P+S) ratio.....	1.29	1.34	1.42	0.97	0.81	0.95
Iodine No., fatty acids	84	83	79	126	121	105
Extractive fraction.						
Total N, per cent of dry gland.....	0.240	0.244	0.248	0.514	0.460	0.490
Ammonia N “ “ “	0.004	0.005	0.006	0.010	0.007	0.008
Pb-precipitable N “ “ “	0.005	0.036	0.018	0.154	0.133	0.128
Basic N “ “ “	0.056	0.051	0.057	0.151	0.164	0.211
Non-basic N “ “ “	0.175	0.152	0.167	0.199	0.156	0.143

* Ovary from which corpus luteum has been dissected.

N sulfuric acid, and the excess acid titrated with 0.02 N sodium hydroxide. The remaining solution was clarified with lead subacetate and nitrogen determined in the filtrate. The filtrate was acidified with sulfuric acid and precipitated with phosphotungstic acid. Non-basic nitrogen was determined in the filtrate from the phosphotungstic acid precipitation.

- The results of these analyses are summarized in Tables III and IV.

DISCUSSION.

The results recorded in Table II show a marked decrease in the amount of estrus-producing hormone in the ovarian blanks and ovarian residue in the later stages of pregnancy. This is due to a diminution not in amount but in activity of the unsaponifiable fat fraction. It was observed at the time of collection of material that the blank ovaries contained many partially developed follicles. The hormone stored in these follicles may account for the greater amount of hormone found in the blanks. If we interpret the increased hormone content of these glands in early pregnancy as indicating increased rate of hormone production, it is difficult to reconcile our observations with those of Frank and Goldberger (9) who found no hormone in the blood in early pregnancy but always found it to be present in the later stages. It appears that either the amount of hormone found in the gland is not indicative of the rate of hormone production, or that some other tissue is also producing this hormone. It is also possible that the rate of storage in the placenta may be a factor in governing the concentration of the hormone in the blood at any particular time. Since the production of the estrus hormone is at present considered not to be a function of the corpus luteum, the increased content of estrus-producing hormone in this gland in later pregnancy is very likely due to storage. Attempts to measure the estrus-inhibiting action of the corpus luteum were unsuccessful. The injection into mice of the unsaponifiable fat fractions of the corpus luteum in quantities equivalent to 50 gm. of fresh gland failed to prevent or delay the occurrence of estrus.

The chemical analysis of the ovarian residue summarized in Tables III and IV shows very little change in this gland during pregnancy. On the other hand, the lipids of the corpus luteum

show evidence of change. The decrease of phosphorus and sulfur in the purified lipid fraction indicates a diminution of phosphatides and sulfatides during pregnancy. Since the total fat content of the gland remains unchanged, we are observing a fatty degeneration of the corpus luteum characterized by a substitution of neutral fat and fatty acids for the more complex lipids. The extractive fractions of the corpus luteum show an increase in basic nitrogen and a corresponding decrease in non-basic nitrogen as pregnancy progresses. In no case have we obtained any evidence of a transfer of lipids between the ovarian residue and the corpus luteum. If we interpret the fatty degeneration of the corpus luteum as evidence of a diminished function, the results are in accordance with the greater physiological importance of this gland in early pregnancy. The fact that the late removal of the corpus luteum does not terminate pregnancy indicates that the protective function of the corpus luteum diminishes in the later stages of pregnancy.

The authors gratefully acknowledge their indebtedness to Dr. David Klein of The Wilson Laboratories, and to Dr. A. A. Swain of Wilson and Company, whose cooperation has made possible the fractional collection of the ovaries.

SUMMARY.

Cow's ovaries have been collected at the early, middle, and late stages of pregnancy and separated into three fractions: (1) blank ovaries without corpora lutea, (2) ovarian residue from which corpora lutea have been dissected, and (3) the dissected corpora lutea. Aliquots of each of these fractions have been analyzed quantitatively for the estrus-producing hormone, by the spayed rat test. The remainder of the ovarian residue and corpus luteum fractions has been used for a chemical examination of the lipids. During pregnancy the estrus-producing hormone diminishes in the ovarian blanks and ovarian residue and increases in the corpus luteum. The increase in the hormone content of the corpus luteum is likely due to storage rather than to production. No chemical changes were found in the ovarian residue. A diminution of phospholipids and sulfolipids and an increase in neutral fat occur in the corpus luteum in the later stages of pregnancy. No change

was observed in cholesterol and cholesterol ester. The fatty degeneration of the corpus luteum parallels its loss in function.

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CHEMICAL STUDIES ON THE SPLEEN.

IV. EVIDENCE FAVORING THE FORMATION OF A COLORLESS FORM OF HEMOGLOBIN AFTER SPLENECTOMY.

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The previous paper of this series (Ray, 1928) pointed out that among the results of splenectomy in the dog there is an accumulation of a hemoglobin derivative incapable of combining with oxygen. This non-functional form reaches a concentration of 20 to 30 per cent of the total pigment, and its appearance in the blood lasts over 6 months. The only information as to the nature of this pigment given at that time was the fact that it could be converted to methemoglobin by the action of potassium ferricyanide. In fact, owing to the unknown nature of the non-functional pigment, the tests were purposely non-specific. Stimson (1927) has made similar observations on the rabbit and her experiments throw still more light on the chemical nature of the non-functional pigment. In place of the Stadie method for the determination of the unknown pigment she used the Van Slyke method of methemoglobin determination. The fact that this method could be applied to this type of determination indicates clearly that the non-functional form may be reduced to functional hemoglobin by the action of sodium hydrosulfite, thus showing the non-functional form to be an oxidation product of hemoglobin. This would lead one to suspect the presence of methemoglobin. Our own spectroscopic observations on dog blood failed to reveal methemoglobin. Stimson carried out observations on rabbit blood by means of the spectrophotometer and measured the absorption at 540 and 560 $m\mu$. From these measurements the ratio of extinction coefficients was computed and found to be

* Crile Scholar.

1.60 or above, a figure which agrees with the observations of H&ri (1917) on oxyhemoglobin.

The spectrophotometric observations recorded in this paper have been made on the blood of the dog following the removal of the spleen and, as will be seen, represent concentrations of unknown pigment varying from 5 to 29 per cent. Unfortunately Stimson's observations had to be made on blood samples with low concentrations, generally less than 10 per cent. We have further determined the light absorption over the important range (500 to 600 $m\mu$) in order that any possible deviation from the normal absorption curve of hemoglobin could be made evident. Further comparisons were made with normal blood and with blood containing an amount of methemoglobin comparable to that of non-functional pigment.

The technique of these experiments, other than that involving the use of the spectrophotometer, was the same as that employed in the other papers of this series. Blood was drawn from the jugular of an unanesthetized dog into a syringe containing potassium oxalate. The functional hemoglobin was determined by the constant volume Van Slyke apparatus and the total pigment by means of the Stadie cyanhemoglobin method. The difference between these two readings is, as in the previous paper, called the non-functional pigment.

The spectrophotometric readings were made with a Keuffel and Esser color analyzer, which we found to be very convenient and accurate. The figures given in this paper are characteristic and are representative of the readings of two observers. The specimen tube length throughout was 10 mm. and the photometer was set to permit only 25 per cent of the light passing the standard tube to enter the spectrometer. This arrangement permits readings as low as 0.25 per cent transmission. We estimate the error of any readings in the range of maximum absorption to be about 2 per cent with this setting. The standard dilution of the blood samples was 1:100 in all cases, with 0.4 per cent NH_4OH as a diluent. In any experiment dilutions were made in such a manner that each flask contained the same concentration of total pigment. This point is important as will be seen later. Samples containing methemoglobin were prepared by adding a calculated amount of amyl nitrite to a sample of blood and the resulting methemoglobin measured by the Stadie method.

A comparison of a series of absorption curves is shown in Fig. 1. The readings are plotted in terms of the optical density of the solution; *i.e.*, the negative log of the percentage of light transmitted. Curve A is that found upon examination of the blood

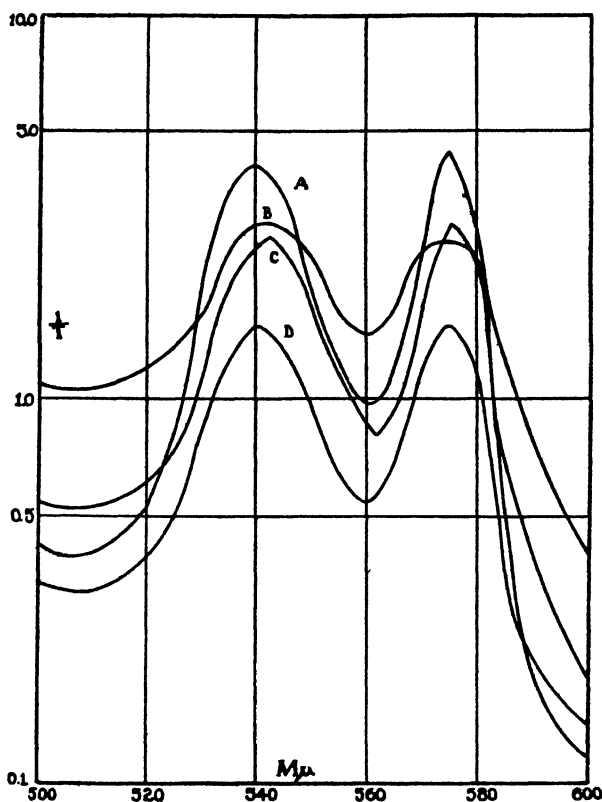


Fig. 1. Comparative absorption curves. Curve A, 17.65 gm. per cent HbO_2 ; Curve B, 17.65 gm. per cent total pigment, 26 per cent MHb; Curve C, 17.65 gm. per cent total pigment, 16 per cent non-functional; Curve D, 17.65 gm. total pigment, 29 per cent non-functional.

of a normal dog. The concentration of hemoglobin in this particular sample was 17.62 gm. per cent, a figure which was used as a basis for adjusting the dilutions of the other curves presented. Curve B is that of the same sample of blood in which 26 per cent

of the hemoglobin had been converted to methemoglobin. The characteristics of this curve are those that might be predicted from the observation of other investigators. In the ranges 500 to 535 and 585 to 600 $m\mu$ the absorption is greater than in the case of hemoglobin alone, while between 535 and 585 $m\mu$ it is less. The absorption bands of hemoglobin are broadened and less well defined due to the presence of methemoglobin.

If Curve C, that found in the case of blood having a non-functional pigment content of 16 per cent, is compared with the foregoing curves, it is obvious that the form of this curve bears a close resemblance to that of oxyhemoglobin. The chief variation is the reduction in absorption in the middle portion of the curve; *i.e.*, 525 to 580 $m\mu$. On the other hand there are distinct differences from the methemoglobin curve, which are sufficiently marked to dismiss methemoglobin as the substance appearing after splenectomy. Curve D, that from a blood sample containing 29 per cent non-functional pigment, shows a similar change to that observed in Curve C, but has a more marked depression of absorption. It will be seen that Curve C is about midway between Curves A and D.

This proportionality of the reduction of absorption to the concentration of non-functional pigment is most evident if we compare the ratio of the concentrations with the ratio of the decrease in absorption in Curves B and C. Taking the curve of oxyhemoglobin as a standard, we find that the optical density of the blood containing 16 per cent non-functional pigment is 13.5 less than the standard, while that of the 29 per cent sample is 24.5 less. For the ratio of 135:245 we have a value of 0.551. For the ratio of concentrations of non-functional pigments, *i.e.* 16:29, we find a value of 0.550. It is obvious that the reduction in light absorption and the reduction in function of hemoglobin are exactly proportional.

The exact relationship of these results arouses interest as to the proportionality of the absorption to the concentration of oxyhemoglobin. The relationship expressed in the preceding paragraph leads to the hypothesis that the observed spectrophotometric curves represent the absorption curves of oxyhemoglobin concentrations found from the oxygen capacity determinations. There are two ways of testing this hypothesis. One, used ex-

tensively by Huefner (1894), is very simple and consists in taking the ratio of the extinction coefficients at 540 and 560 $m\mu$. The most recent figure for this ratio is that given by Hári (1917), namely 1.60. This ratio simply states whether or not the sample under consideration is pure oxyhemoglobin, since this ratio is a constant for any given substance. The second method is more useful for our purpose in that it enables us to determine the actual concentration of oxyhemoglobin. It consists in calculating the concentration of hemoglobin from the extinction coefficient and a constant, the absorption ratio.¹ In this research both methods were employed and the results are given in Table I.

TABLE I.

Comparison of Oxyhemoglobin with Blood Samples Containing Varying Amounts of Non-Functional Pigment.

Each dilution flask contained 0.01765 gm. of total pigment.

Sample.	E_1 (1)	E_2 (2)	$E_1:E_2$ (3)	Hb ₁ (4)	Hb ₂ (5)
HbO ₂	1.602	0.977	1.63	17.65	17.62
16 per cent non-functional pigment..	1.423	0.908	1.59	14.81	15.36
29 " " " " ..	1.190	0.742	1.60	12.54	12.87

E_1 = extinction coefficient, $-\log T$ at 560 $m\mu$.

E_2 = " " " " " " 540 "

A = absorption ratio at 542 $m\mu$ = 0.0011 (Davis and Sheard, 1927).

Concentrations have been calculated from the usual formula, $C = 100 A, D, V, E$, where C = concentration in gm. per cc., D = thickness of the absorbing layer, V = volume of diluted blood, $E = -\log T$ at 542 $m\mu$, Hb₁ = HbO₂ from O₂ capacity, Hb₂ = HbO₂ from spectrophotometer.

The ratios of extinction coefficients in the case of altered blood, given in Column 3, show an excellent agreement with those derived from pure oxyhemoglobin. That found for oxyhemoglobin in our case is 1.63, rather higher than usual, but within the error of the method. Both of the blood samples from splenectomized animals show as close an agreement. As far as these data reveal

¹ The accuracy of the spectrophotometric determination is shown by the agreement of the value A (absorption ratio) found in our readings as compared with that found by Davis and Sheard (1927). These authors found $A = 0.001100 \pm 0.000003$ for an average of fifteen determinations. We find $A = 0.001113$, which is within the deviation of readings (Davis and Sheard).

anything, we are dealing with three solutions of oxyhemoglobin. There is no evidence on the basis of this calculation that any other substance is present.

It remains necessary therefore to apply the second test; *i.e.*, to calculate the concentration of oxyhemoglobin in each solution by means of the extinction coefficient at 542 $m\mu$ and to compare this with the value found by the gasometric measurement. This was done and the results of the calculations are given in Column 5 of Table I. The agreement with the readings made on the Van Slyke apparatus is not absolute in the samples of unknown pig-

TABLE II.

Further Comparison of Oxyhemoglobin Concentrations As Determined by Different Methods (in Gm. Per Cent).

Sample No.	Total pigment.	(HbO ₂) ₁ *	Leucohemoglobin.	Per cent Hb.	(HbO ₂) ₂ †
1	16.22	13.26	2.96	18.3	13.28
2	16.44	13.67	2.77	16.8	13.05
3	16.46	12.93	3.53	21.8	13.05
4	16.35	13.85	2.50	15.3	13.05
5	22.22	19.75	2.47	11.1	18.22
6‡	16.72	14.95	1.77	10.5	14.68
7§	7.20	6.09	1.11	15.4	6.10
8	13.80	13.70	0.10	0.72	
9	13.40	13.44	-0.04	-0.33	

* (HbO₂)₁ determined from O₂ capacity.

† (HbO₂)₂ determined from absorption at 542 $m\mu$.

‡ Concentrated by centrifugation.

§ Diluted.

ment, the difference being 3.3 per cent in that with 16 per cent non-functional hemoglobin, and 2.6 per cent in the other sample. These deviations are not unduly large when one considers the manipulation of the blood before the spectrophotometric determination. In spite of this deviation the results of the spectrophotometer determination agree with the Van Slyke rather than with the colorimetric method. It will be recalled that the three flasks in this experiment were made up to the same concentration on the basis of the total pigment determination. In spite of this we are able to predict, within the possible error of the method, the concentration of oxyhemoglobin determined by the gaso-

metric method. The agreement between the concentration of hemoglobin as determined from the oxygen capacity and that determined by the spectrophotometer in other experiments is shown in Table II. Blood samples showing a wide range of non-functional "pigment" have been compared by means of the three types of measurement. In every case the hemoglobin found spectrophotometrically agrees more closely with that found by the Van Slyke apparatus than that found by the colorimeter. The last two dogs are control animals (hysterectomy). They show a close agreement between the Stadie determination and the gasometric determination in the unsplenectomized animal.

As a result of these calculations we are forced to but one conclusion. It is apparent that the non-functional hemoglobin has no effect on the absorption curve of oxyhemoglobin present in the solution.²

One must, however, consider what known blood pigments might produce such a variation in the Stadie determination yet cannot be determined by the spectrophotometer. Hematin is, perhaps, the most common substance capable of acting in this manner. Conant and Fieser (1925) have called attention to the fact that the presence of hematin interferes with the Stadie determination, while Haurowitz (personal communication) claims that hematin cannot be detected in the presence of oxyhemoglobin by means of the spectrophotometer. It is possible that in the absence of the spleen red blood cell destruction takes place intravascularly with hematin as an intermediary between hemoglobin and bilirubin (Mann, Sheard, Bollman, and Baldes, 1926).

We have carried out experiments to determine the influence of hematin on the absorption curve of oxyhemoglobin. In order to determine any possible effect of the presence of hematin the following procedure was adopted. Two solutions were employed, oxyhemoglobin (laked red blood cells) and alkaline hematin. These were mixed in varying concentrations as shown in Table III. Each mixture was diluted 1:100 with 0.4 per cent NH_4OH and examined by the spectrophotometer and the absorption curve plotted. These curves are shown in Fig. 2. Examination of these curves shows a definite change from the absorption curve of

² In certain experiments the observations were extended to 450 and 650 $m\mu$ with no obvious deviation.

oxyhemoglobin. That this effect is due to the presence of a second pigment and is not the result of dilution may be demonstrated by computing the change in the value of the ratio of the absorption at 540 $m\mu$ to that at 560 $m\mu$. We have already pointed

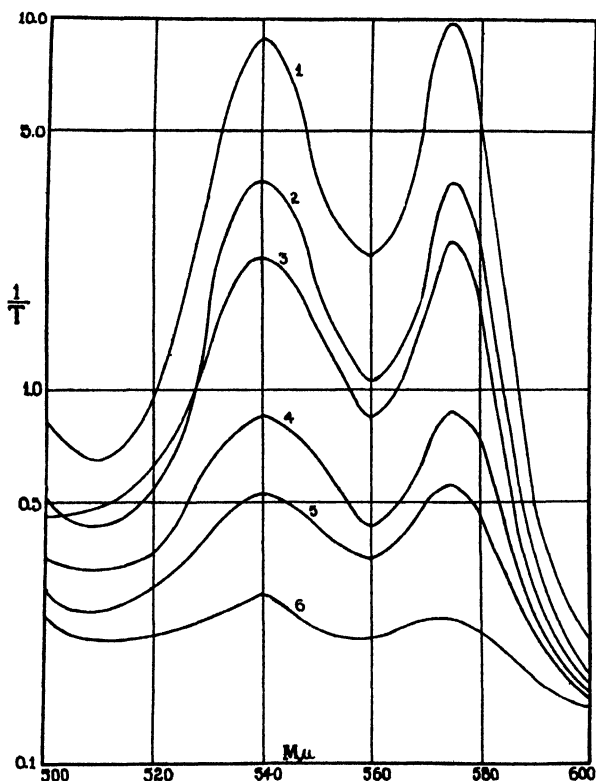


FIG. 2. Comparative absorption curves of increasing proportions of hematin added to HbO_2 . The figures refer to sample numbers as given in Table III.

out that in the case of oxyhemoglobin this ratio equals 1.60. Fig. 3 shows that the magnitude of the ratio decreases directly with increasing amounts of hematin. It is obvious that hematin could not have been present in the blood of our splenectomized dogs, since the ratios given in Table I are those of pure oxyhemoglobin.

Other hemoglobin derivatives must also be ruled out; namely, cathemoglobin, hematoporphyrin, hemochromogen, etc. If one studies the position of the absorption bands of these various com-

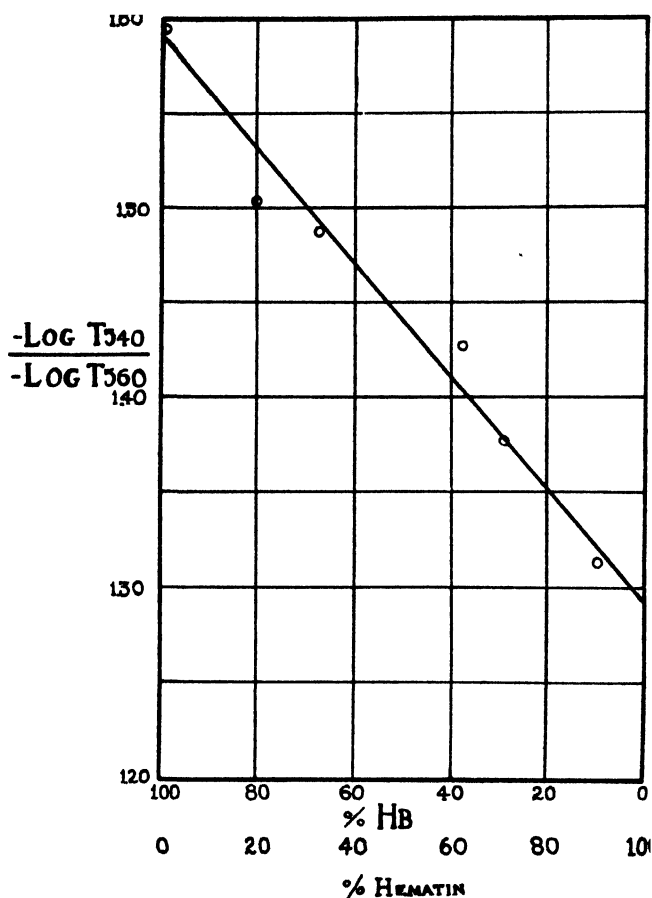


FIG. 3. Changes in the value of the ratio of the absorption at 540 $m\mu$ to that at 560 $m\mu$, occurring as a result of increasing the amount of hematin in an HbO_2 solution.

pounds it is quite obvious that the presence of any one would affect the absorption curve of oxyhemoglobin.

Having thus eliminated the known colored derivatives of hemo-

globin, we can only suggest that the non-functional pigment is of a hitherto unrecognized form, which is colorless. Because of the ease with which it is determined by the Stadie test, a close relationship to hemoglobin or methemoglobin may be supposed. This is in agreement with a statement by Conant and Scott (1928) to the effect that if an intermediate compound exists between hemoglobin and methemoglobin it cannot be detected by means of the spectrophotometer.

TABLE III.

Composition of Mixtures of Hemoglobin and Hematin Used for Spectrophotometric Examination.

Sample No.	Hb	Hb	Ht*	Hb = Ht	Total pigment.	Ht
	per cent	gm. per cent	gm. per cent	gm. per cent	gm. per cent	per cent
1	100	10.59	0.00	0.00	10.59	0.0
2	85	9.00	0.05	1.71	10.71	16.0
3	70	7.41	0.09	3.42	10.83	31.6
4	50	5.29	0.15	5.70	10.99	51.9
5	30	3.18	0.21	7.98	11.16	71.4
6	15	1.59	0.25	9.68	11.27	86.0
7	0	0.00	0.30	11.45	11.45	100.0

* Ht represents hematin.

We tentatively suggest, therefore, that this unknown non-functional form of hemoglobin be known as "leucohemoglobin."

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COMPARATIVE STUDIES OF THE METABOLISM OF AMINO ACIDS.

III. THE FORMATION OF GLYCOGEN AFTER ORAL ADMINISTRATION OF AMINO ACIDS TO WHITE RATS.*

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That glycogen can be formed from protein in the animal organism has long been accepted (2). Pfüger in his classical monograph on glycogen (3) has pointed out many errors in the older work and has concluded: "Ich kann diese Auffassung nicht für falsch erklären, sie ist noch nicht bewiesen."¹ However, in 1910, after a long series of carefully controlled experiments (4), he concluded that protein was a possible source of glycogen. Important also are the observations of Osborne and Mendel (5) in which the glycogen contents of the bodies of rats maintained over long periods of time on diets entirely devoid of preformed carbohydrate were found to be similar although slightly less than those of control rats which received the usual starch-containing food mixtures.

With the acceptance of the theory that the behavior of protein in intermediary metabolism is determined by the chemical transformation of its component parts, the individual amino acids, it is evident that the question of the various amino acids as sources of glycogen becomes of great significance. It has long been recognized that, in the phlorhizinized dog, the conversion of certain amino acids to glucose may occur (6, 7). However, the demonstration that under the pathological conditions of experimental

* The material present in this and the preceding paper of this series (1) represents an abstract of part of the thesis presented by Robert Hugh Wilson in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University of Michigan.

¹ Pfüger (3) p. 316.

diabetes, conversion to glucose is accomplished does not necessarily have important bearing in a consideration of the normal paths of metabolism of the amino acids in the organism. Similar criticism may be made of the application of the results of Mann and his coworkers (8) with hepatectomized dogs to the interpretation of normal metabolic phenomena.

The question of glycogen formation after the administration of amino acids to normal fasted animals has not been extensively studied. Cohn (9) in an attempt to establish the validity of the theory of carbohydrate formation from amino acids containing 6 carbon atoms observed that the livers of fasted rabbits which had received leucine orally contained more glycogen than did those of control animals. Simon (10) in a repetition of this work in which the animals were rendered glycogen-free by the use of strychnine and fasting, could find no evidence of glycogen formation either in the liver or in the rest of the body. Neuberg and Langstein (11) fed large amounts (20 to 30 gm.) of alanine to fasting rabbits and found 1 to 2 gm. of liver glycogen, from which they were led to consider alanine as a source of glycogen. No details are given nor are any control experiments cited. It can hardly be said from the data presented that the formation of glycogen from alanine has been definitely established, since liver values as high as those reported by Neuberg and Langstein have been observed by other investigators in rabbits fasted for several days without other treatment. Pflüger (12) fed glycine to two fasting dogs which had been depleted of glycogen by injections of phlorhizin. In one animal, no increase in liver glycogen was observed; in the other, 4.47 per cent glycogen was present. In two other experiments in which no phlorhizin was used liver glycogen values of 1.70 and 1.58 per cent were noted after glycine feedings.

Evidence that a mixture of amino acids could form glycogen when fed to a fasting dog was presented by de Corral (13) on the basis of changes in the respiratory quotient. The respiratory quotient was observed to be considerably higher 18 to 20 hours after feeding the amino acid mixture than was to be expected if protein were being oxidized, which was interpreted as evidence of the formation and combustion of glycogen.

Casein supplemented by alanine was found to be more effective in the formation of liver glycogen in white rats than was casein

alone (14), while if glutamic acid were fed with casein, glycogen formation appeared to be checked. Unfortunately no adequate control experiments on fasting animals were presented.

Thus it is evident that of the various investigations which are concerned with glycogen formation after the oral administration of amino acids, only two appear to have been well controlled, that of Pflüger (12) with glycine and that of Simon (10) with leucine. It was felt that these experiments might well be repeated and extended with animals in which the extent of the absorption of amino acids had also been studied (1) with uniform experimental conditions and with a series of animals sufficiently large to eliminate as far as possible errors due to individual variations. A possible relationship between the amount and rate of absorption of the amino acids and the changes in the glycogen content might exist.

Rats appear to be more suitable for the study of glycogen formation than are the common large experimental animals of the laboratory. Hens, rabbits, and dogs often show considerable amounts of glycogen after fasts of several days. The body of the rat, on the other hand, contains little glycogen at the end of a 24 hour fast and the fasting glycogen levels are much more uniform. Barbour, Chaikoff, Macleod, and Orr (15) found an average liver glycogen content of 0.16 per cent in white rats after a 24 hour fast with values ranging from 0.10 to 0.31 per cent in one series and from 0.08 to 0.48 per cent in a second series. Cori and Cori have reported two series of glycogen determinations in rats after 24 hour fasts. In the first series of sixteen animals (16) an average liver glycogen content of 0.20 per cent was observed, while in a second series of eight animals, the average value obtained was 0.10 per cent. Variations found by Cori and Cori were similar to those noted by the Toronto observers (15). After a 24 hour fasting period, Cori and Cori also noted that the glycogen content of the entire animal averaged 0.143 per cent of the body weight. These average figures and variations are similar to those we have obtained in the present series.

EXPERIMENTAL.

For this study of glyconeogenesis from amino acids, the same rats which were used for the study of the rate of absorption from

the intestine (1) were employed for the most part. The general technique for the preparation of the animals, *i.e.* the period of fasting and the oral administration of the amino acids, was the same as already reported, except that in all cases in which glycogen formation was studied, the preliminary fast was of 24 hours duration. At the end of the period of absorption under study, the animals were killed either by a blow on the head or with chloroform, the intestines were removed, the liver and the rest of the body weighed as quickly as possible and covered with a hot saturated solution of sodium hydroxide. In the earlier experiments, in order immediately to check glycogenolysis, the livers and bodies of the animals were frozen with liquid air. Later this was discontinued since it was found that in the time elapsing between the slaughter of the animal and the heating with the strong alkali, no noticeable destruction of glycogen occurred. The glycogen was precipitated from the hydrolyzed tissue by the addition of 2 volumes of 95 per cent alcohol. After careful washing, the precipitated glycogen was hydrolyzed with hydrochloric acid, the solution carefully neutralized with sodium hydroxide, and the glucose was determined by the method of Hagedorn and Jensen, checked in some cases by determination by the Munson and Walker modification of the Bertrand method.

In the experiments with fasted animals, to which no amino acids were fed, the rats, after the preliminary fasting period, received 2 cc. of water by stomach tube in place of the amino acid solution. The average values, 0.21 per cent in the liver with individual values ranging from 0.12 to 0.33 per cent, and 53 mg. per 100 gm. in the whole body with values ranging from 28 to 77 mg. were fairly uniform, particularly in the case of the liver. These values for liver glycogen agree well with those of Cori and Cori (16, 17) but the content of glycogen of the entire body as found by us was somewhat lower than the figure (0.143 per cent) found by them.

In later experiments with amino acids, it was desirable to feed the rats by stomach tube several times during the day, in an attempt to keep a supply of amino acids constantly present in the intestine in order to obtain a maximum absorption over a longer period. In order properly to control these experiments, rats were fed 2 cc. of water at 4 hour intervals and killed after 16 hours. No

significant increases in the glycogen content resulted, although in two of the seven experiments of this kind, the liver glycogen values were slightly increased.

In preliminary experiments, mixtures of protein (casein or gelatin) or glycine with butter² were placed in the cages and the rats were permitted to eat at will for 24 to 30 hours. Any change in the glycogen content of the animals was considered to be due to the effect of the protein or glycine since it has been shown pre-

TABLE I.

Glycogen Content of Rats When Fed Solid Protein or Glycine and Butter Oil.

Substance fed.*	Absorption time.	Glycogen.	
		Liver.	Entire body.
	hrs.	per cent	mg. per 100 gm.
Casein and butter.	29	1.67	220
	30	1.36	199
	30	1.97	228
Average.		1.67	216
Gelatin and butter.	24	0.40	157
	25	0.72	143
	25	0.68	113
	26	1.86	149
	26	2.06	205
Average.		1.14	153
Glycine and butter.	26	0.89	129
	25	0.77	150
Average.		0.83	140

* In each of these mixtures the butter constituted 25 per cent of the weight, the protein or glycine the remainder.

vously (18) that under these experimental conditions the butter did not contribute significantly to the glycogen store. The results (Table I) show that these animals had a definitely higher percentage of glycogen than did the controls, thus confirming previous workers.

² Since it was impossible to secure consumption of the proteins or glycine alone, 25 per cent of purified butter oil was added. These mixtures were eaten well. It was difficult, however, to measure exactly the food consumed due to the loss by scattering.

TABLE II.

Glycogen Content of Rats after Feeding dl-Alanine, d-Alanine, and dl-Alanine as the Sodium Salt by Stomach Tube.

	Absorption time.	Amount absorbed.	Glycogen.	
			Liver.	Entire body.
	hrs.	mg. per 100 gm.	per cent	mg. per 100 gm.
dl-Alanine.	2	168	0.42	88
	2	115	0.36	93
	2	183	0.81	95
	2	150	0.37	84
Average.			0.49	90
dl-Alanine.	3	248	0.28	60
	3	191	0.53	65
	3	135	0.29	66
	3	266	0.23	48
	3	200	1.38	108
	3	248	0.39	62
	3	224	1.07	
Average.			0.87	
Average.			0.63	67
dl-Alanine.	4	302	0.98	99
	4	285	0.47	77
Average.			0.73	88
dl-Alanine.	15		1.58	165
	15		0.59	91
	16		1.21	147
Average.			1.13	134
d-Alanine.	3	273	1.25	129
	3	236	0.73	89
	3	224	0.39	101
	3	222	0.43	76
	3	211	0.61	92
	3	232	0.63	103
Average.			0.67	98
dl-Alanine as the Na salt.	2	107	0.33	78
	2	97	0.29	
	2		0.57	
	2	63	0.18	
	2	93	0.57	
Average.			0.39	78

The glycogen values obtained after oral administration of the amino acids are presented in Tables II and III. Administration of alanine (*d*-, *dl*-, and the sodium salt of *dl*-alanine) resulted in

TABLE III.

Glycogen Content of Rats after Feeding Amino Acids by Stomach Tube.

Amino acid fed.	No. of animals used.	Time of absorption.	Amount absorbed, range.	Glycogen.			
				Liver.		Entire body.	
				Range.	Average.	Range.	Average.
		hrs.	mg. per 100 gm.	per cent	per cent	mg. per 100 gm.	mg. per 100 gm.
Glycine.	3	1-2	107-117	0.10-0.22	0.16	53-81	80
	5	3	127-167	0.10-0.29	0.20	50-82	61
	2	4	161-197	0.16-0.25	0.21	71-103	87
	5	15-16		0.13-0.46	0.26	29-100	69
Glycine (Na salt).*	3	1	40-85	0.14-0.17	0.15	41-58	50
	5	2	116-127	0.07-0.25	0.16	32-56	45
	2	3	186-192	0.20	0.20	68	68
	1	4	231	0.15	0.15	62	62
Leucine (Na salt).	3	2	49-94	0.15-0.21	0.17	50	50
	5	3	102-172	0.14-0.24	0.19	31-67	45
	2	4	156-184	0.21-0.24	0.23	31-70	51
	3	11-16		0.10-0.23	0.14	24-50	41
Glutamic acid (Na salt).	3	1	77-116	0.19-0.22	0.21	54-75	67
	4	2	114-207	0.10-0.39	0.25	35-77	51
	7	3	158-222	0.21-0.59	0.33	54-95	72
	4	4	233-290	0.15-0.33	0.22	48-70	62
	2	15-16		0.23-0.38	0.31	67-98	83

* Only half of the amount of sodium hydroxide necessary to neutralize the carboxyl groups of alanine or glycine was added (1). Leucine was fed as the sodium salt and glutamic acid as the monosodium salt. In the case of these last two amino acids, it was impossible to prepare solutions of the free amino acids sufficiently concentrated for the purposes of the experiments.

definitely higher levels of glycogen (Table II), both for the liver and for the total glycogen content of the body. The increases in the total glycogen content were due in part to the higher glycogen content of the liver, but in most cases, the rest of the body also

showed an increase. Thus in the control series, the absolute values for glycogen of the body without the inclusion of the liver ranged from 29 to 99 mg. with only ten of the values of a series of twenty-five exceeding 70 mg., while after the administration of *dl*-alanine, the absolute values ranged from 39 to 118 mg. with eleven values of a series of sixteen exceeding 70 mg.

The results obtained after the administration of the other amino acids studied (Table III) do not indicate any distinct changes in the glycogen content either of the liver or of the entire body. The negative results with leucine are in accord with the earlier work of Simon (10) and opposed to the results of Cohn (9) who observed deposition of glycogen after the administration of leucine.

After the ingestion of monosodium glutamate, certain of the values for liver glycogen were slightly but clearly greater than those of the control rats. There were also some low values, but these did not occur as frequently as in the control series. The figures for the glycogen content of the entire body were slightly greater than the average for the controls, but the difference was not marked. After the longer periods (16 hours) of absorption, the glycogen values were within the range found for control rats similarly treated. It may be noted that considerable difficulty was experienced in the absorption experiments with glutamic acid over these longer periods of time. In two experiments the rats died during the course of the period of absorption and a third appeared ill during the last 6 hours of the experiment. We have observed previously this toxic action of glutamic acid (19) and can offer no explanation for it.

Certain previous investigations have suggested that glyconeogenesis may occur more readily from alanine than from glycine or glutamic acid, although all three amino acids have been shown to give rise to urinary glucose in the phlorhizinized dog (6, 7, 20). Voegtlin, Dunn, and Thompson (21) believed that alanine was more rapidly converted to glucose than was glycine or glutamic acid. They fed rats, previously injected with minimal lethal doses of insulin, the amino acids by stomach tube. Ingested alanine alone was able to prevent insulin convulsions and the death of the animal. The protective action of glycine or of glutamic acid was slight. It was concluded that alanine was absorbed much more rapidly than were the other amino acids or

that it was more easily transformed to glucose. Alanine protected the insulin-injected rats almost as effectively as did glucose, which suggests similar rates of absorption. But, as previously discussed (1), glucose appears to be absorbed much more rapidly than alanine. It may be that with the larger amounts of alanine fed by Voegtlin and coworkers (6 to 7 gm. per kilo of body weight), the absorption of alanine was greater than that observed by us.

A difference in the amount of glucose which could be formed during a given period of time might be expected even if glycine and alanine were converted to glucose with equal ease. We have shown previously that alanine is absorbed more rapidly than glycine (1). On the assumption of a complete utilization of all 3 carbon atoms of alanine for glucose formation, the glucose formed from alanine would amount to 101 per cent of the weight of the alanine. On the other hand, if all the carbon of the glycine molecule is available for glucose formation (6), which has been denied by Cremer (22), the glucose formed would be equal to only 80 per cent of the weight of the glycine converted. Hence it follows that from glycine with an average rate of absorption of 50 mg. per 100 gm. of body weight per hour (1), the maximum amount of glucose formed per 100 gm. of body weight would be 40 mg. per hour, while from alanine with an absorption rate of 73.6 mg., glucose formation might occur at the rate of 74 mg. per hour per 100 gm. of rat. The same type of calculation for glutamic acid, which has been shown to form glucose in phlorhizin diabetes to the extent of 3 of its 5 carbon atoms (23) indicates that the maximum formation of glucose from this amino acid would be about 37 mg. per 100 gm. per hour. It is clear that, on a theoretical basis, the opportunity for the formation of glucose should be much greater after the ingestion of alanine than after either glycine or glutamic acid.

It is possible also that the increased metabolism occasioned by the administration of the amino acids (specific dynamic action) may also affect the deposition of glycogen. The increased metabolism is indicative of increased oxidation and since carbohydrate is the most readily oxidized of the organic foodstuffs, it might be expected that to furnish the energy for this increased metabolism, glucose, if available, would be burned. It has been found that alanine increases metabolism to the extent of about

69 per cent of the increase which results from the ingestion of an equal weight of glycine (24). Hence less of the glucose formed from alanine should be used for purposes of oxidation than would be the case after the ingestion of glycine. This would leave more glucose available for deposition as glycogen after the administration of alanine.

A consideration of these factors, more rapid absorption of alanine, the possible greater ease of its conversion to glucose, and its lower specific dynamic action, indicates that formation of glycogen from alanine might be expected to take place more readily than from glycine.

In conclusion it should be pointed out that the results of the present study are not to be considered as significant except over short periods of time. They indicate that the formation of glycogen occurs more readily from alanine than from glycine, glutamic acid, or leucine. Over longer periods of time with the continued ingestion of large amounts of the amino acids, as for example in the experiments of Pflüger (12), glycogen formation from other amino acids may well be observed. Further studies to include other amino acids and a wider variety of conditions are in progress.

SUMMARY.

1. The glycogen contents of the liver and the entire body except the abdominal viscera of rats fed casein, gelatin, or glycine with butter for 24 to 30 hours were higher than those of the fasted control rats.

2. The oral administration of *D*- and *DL*-alanine by stomach tube to fasting white rats resulted in a rapid deposition of significant amounts of glycogen. After similar administration of glycine and *L*-leucine, the glycogen contents were similar to those of the control animals. The ingestion of the monosodium salt of *D*-glutamic acid was followed by a slight increase in the glycogen content as compared with control animals.

3. The possible relationships of the rates of absorption and specific dynamic actions of the amino acids to their ability to serve as precursors of glycogen are discussed.

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STUDIES IN CARBOHYDRATE METABOLISM.

I. A GLUCOSE-LACTIC ACID CYCLE INVOLVING MUSCLE AND LIVER.*

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As a result of the important researches of Meyerhof and Hill we are in possession of a picture of the mechanism of muscular contraction, a mechanism which depends in a large measure on the reversible reaction between glycogen and lactic acid. The energy for the contraction of muscle is afforded by the breakdown of glycogen to lactic acid. During the recovery phase of muscular contraction oxidations supply the energy necessary for the rebuilding of lactic acid to glycogen. Meyerhof (1920) demonstrated that a large part of the lactic acid formed in the contraction of excised muscle of frog is resynthesized to glycogen during the recovery from work. Similarly, Hill, Long, and Lupton (1924-25) concluded from studies on human beings that the greater part of the lactic acid produced during exercise was reconverted to glycogen, for oxidations could account for the disappearance of but a small portion of this lactic acid. It is of interest to determine where in the intact mammal the reconversion of lactic acid to glycogen occurs. Since lactic acid escapes from the exercising muscles to the blood, all parts of the body are exposed to increased concentrations of lactic acid. Is this lactic acid synthesized to glycogen by organs other than muscle?

There is direct evidence demonstrating that the liver may remove lactic acid from the blood and retain that substance as

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glycogen or release it to the circulating fluid as glucose. Cori and Cori (1929) fed lactic acid to rats and noted glycogen formation in the liver, and it has been known since the time of Claude Bernard that the liver supplies glucose to the body. More recently Mann and Magath (1922) have shown that the liver is the chief source of the glucose of the blood stream.

There is also indirect evidence of such action by the liver. Von Noorden and Embden (1906) asserted that after extirpation of the liver, the blood sugar decreases while lactic acid accumulates, and believed that the liver was the site of the formation of glucose from lactic acid. Parnas and Baer (1912) perfused a solution of lactic acid through excised turtle liver and observed an increase of the glycogen content of that organ. Perfusion studies were also made by Barrenschenn (1914), Baldes and Silberstein (1917), and Burn and Marks (1926). Although Burn and Marks (1926) did not find that all the glucose released by the liver could be accounted for by the lactic acid removed by that organ, results of the other authors obtained on excised livers of normal, depancreatized, and phlorhizinized dogs indicated that the glucose which appeared in the perfusion system did arise from lactic acid. These results probably explain the experiments of Embden and Salomon (1905), Mandel and Lusk (1906), and Parnas and Baer (1912), in which lactic acid, injected in depancreatized or phlorhizinized animals, reappeared as glucose in the urine. If the liver removes lactic acid from the blood and supplies glucose to the circulating medium, it seems possible that in the intact mammal, the liver is a site of recovery. In order to test this hypothesis the blood entering and leaving muscle and liver during rest and exercise of amytalized and decerebrate dogs was analyzed for lactic acid and glucose.

Method.

The femoral vessels of dogs were exposed in the femoral triangle and a longitudinal abdominal incision was made. These operations permitted samples of blood to be drawn practically simultaneously from the femoral artery, femoral vein, portal vein, and hepatic vein, during rest and during moderate exercise produced by induction shocks at the rate of one stimulus per second. The lactic acid content of the blood samples was estimated according

to the procedure of Friedemann, Cotonio, and Shaffer (1927), and glucose by the Shaffer-Hartmann (1920-21) and Hagedorn-Jensen methods (1923). Differences of 5 mg. per cent or more in the lactic acid and glucose content of two samples of blood were considered significant.

In all, forty-two dogs, varying in weight from 15 to 29.5 kilos and fasted for periods extending from 1 to 8 days, were studied. Eleven dogs were decerebrated by Schmidt's method (1923) and twenty-nine other animals were amytalized. Eight of the amytalized dogs were eviscerated by Andrews' technique (1927-28). The level of the blood sugar of the eviscerated dogs was maintained by injections of glucose. Two animals, one under the influence of morphine and another studied without any general anesthetic, were subjected only to the exposure of the femoral vessels. Some observations were made incidentally on animals that had been injected with insulin and adrenalin. These experiments are included in Tables I, II, VI, and VII, since the results do not differ significantly from the others. Eight human beings, in the post-absorptive state, were also studied. The blood samples of the human subjects were drawn simultaneously from the brachial artery and the antebrachial vein.

Results.

It is quite impossible because of lack of space to tabulate all the experiments in detail. Tables I and II record the contrasted findings of the lactic acid exchanges of the blood on its passage through muscle and liver. Table I presents typical results. It may be seen (Table I, A) that the concentration of the lactic acid of the blood leaving muscle in the femoral vein was greater than that in the femoral artery, while (Table I, B) the level of the lactic acid of the blood coming from the liver in the hepatic vein was lower than that of the arterial or portal blood entering that organ. It will be noted in Table II, which contains a summary of the results, that in most of the thirty observations on fifteen amytalized dogs and nineteen observations on nine decerebrate animals, muscle added lactic acid to the blood both at rest and during exercise. This action of muscle was least marked in the resting amytalized dogs. On the other hand the liver usually removed lactic acid from the blood as is seen in the twenty-three observa-

tions on nine decerebrate dogs. Only four times did the liver of decerebrate dogs add lactic acid to the blood. This occurred twice in the same animal during exercise and twice in resting animals after the injection of adrenalin, although on two other occasions the injection of adrenalin had no such action. The single observation of a decerebrate dog, disclosing a release of

TABLE I.
Lactic Acid Content of Blood.

The results are expressed in mg. per cent.

Date.		Duration of experiment.	Femo- ral artery.	Femo- ral vein.	
A. Blood during rest and exercise of lower extremities.					
1927					
July 20	Amytal anesthesia.	Exercise. 1 min.	27	35	
		Recovery. 10 "	45	52	
Sept. 23	" "	Rest.	6	10	
		Exercise. 15 min.	21	42	
" 27	" "	Rest.	20	27	
		Exercise. 1 hr., 8 min.	32	37	
Dec. 1	Decerebrate.	Rest.	58	77	
		" 2 hrs.	30	36	
		" 3 "	27	32	
B. Blood entering and leaving liver.					
			Artery.	Portal vein.	Hepatic vein.
July 25	Amytal anesthesia.	Exercise. 5 min.	64	42	24
		Recovery. 5 " after work.	97	60	46
		" 15 " " "	45	34	21
Aug. 25	Decerebrate.	Rest.	79	78	67

lactic acid from the liver, was obtained from an animal in a pre-mortal condition.

Under certain conditions muscle may remove lactic acid from the blood as shown in Table III; the lactic acid escaping from the exercising muscles of the upper extremities was absorbed by the resting muscles of the lower extremities. In the observation

made after 35 minutes of exercise the resting muscles appeared to have been saturated with lactic acid.

The effects of functional removal of the liver were studied in eight dogs. Only two observations are recorded in Table IV, but in every instance a gradual increase in the lactic acid content of the arterial blood was found.

TABLE II.
Changes in Lactic Acid Content of Blood on Passage through Muscle and Liver.

		Muscle.			Liver.		
		Added.*	No change.†	Removed.‡	Added.*	No change.†	Removed.‡
Amytal anesthesia.	Exercise.	5	2	0	2	1	7
	Rest.	12	10	1	2	4	7
Decerebrate.	Exercise.	4	0	0	0	1	1
	Rest.	12	1	2	1	0	8

* Number of cases in which there was an increase of 5 mg. per cent or more of lactic acid.

† Number of cases in which changes were within experimental error.

‡ Number of cases in which there was an absorption of 5 mg. per cent or more of lactic acid.

TABLE III.
Lactic Acid of Blood of Femoral Vessels during Exercise of Upper Extremities.

Date	Anesthetic.	Duration of exercise.	Femoral artery.	Femoral vein.
1937		min.	mg. per cent	mg. per cent
Aug. 6	Amytal.	5	67	60
		15	39	35
		25	79	64
		35	97	112

Table V, which presents the data of a decerebrate dog, discloses that the high levels of lactic acid and glucose resulting from the operative procedures were gradually reduced.

The changes taking place in the glucose concentration of the blood on traversing muscle and liver are presented in Tables VI

and VII. From the typical results of Table VI, A, it may be seen that the glucose content of the blood returning from muscle in the

TABLE IV.

Lactic Acid Content of Arterial Blood after Evisceration, Amytal Anesthesia.

Date.	Time after evisceration.		Lactic acid.
	hrs.	min.	mg. per cent
1928			
Feb. 29		5	50
	1	5	91
	2	15	110
	2	45	117
	3	15	124
	3	45	135
	4	15	136
	5	15	141
	5	45	140
	6	15	146
	6	45	140
	7	10	150
	8	15	148
	9	15	157
	10		190
	11		202
Jan. 17		24	21
	1	24	31
	1	54	56
	2	24	82

TABLE V.

Glucose and Lactic Acid Content of Arterial Blood after Decerebration.

Date.	Time after decerebration.		Lactic acid.	Glucose.
	hrs.	min.	mg. per cent	mg. per cent
1928				
Mar. 7		6	111	199
	1	13	76	176
	2	8	68	149
	4	8	23	138
	6	8	20	120
	8	8	16	95

femoral vein was less than that of the femoral artery, while (Table VI, B) the concentration of the glucose of the blood leaving the liver in the hepatic vein was greater than that of the arterial or

TABLE VI.
Glucose Content of Blood.

The results are expressed in mg. per cent.

Date.		Duration of experiment.	Femo- ral artery.	Femo- ral vein.
A. Blood during rest and exercise of lower extremities.				
1927				
Sept. 23	Amytal anesthesia.	Rest.	116	100
		Exercise. 15 min.	130	114
Aug. 23	Decerebrate.	" 15 "	154	131

B. Blood entering and leaving liver.

			Artery.	Portal vein.	Hepatic vein.
July 25	Amytal anesthesia.	Exercise. 5 min.	125	128	160
		Recovery. 5 " after work.	144	143	161
		" 15 " " "	134	138	156
Nov. 18	Decerebrate.	Rest.	259	240	274

TABLE VII.
Changes in Glucose Content of Blood on Passage through Muscle and Liver

		Muscle.			Liver.		
		Added.*	No change.†	Removed.‡	Added.*	No change.†	Removed.‡
Amytal anesthesia.	Exercise.	0	2	6	4	1	1
	Rest.	3	4	11	11	0	0
Decerebrate.	Exercise.	0	0	1	2	0	0
	Rest.	1	3	9	7	4	0

* Number of cases in which there was an increase of 5 mg. per cent or more of lactic acid.

† Number of cases in which changes were within experimental error.

‡ Number of cases in which there was an absorption of 5 mg. per cent or more of lactic acid.

portal blood. From the summary (Table VII) it may be noted in the twenty-six observations on thirteen amytalized dogs, and

thirteen observations on seven decerebrate animals, that muscles usually removed glucose from the blood. While in direct contrast it was seen in seventeen observations on eight amygalized dogs and thirteen observations on seven decerebrate animals, that the liver usually added glucose, and did this, moreover, irrespective of the level of the blood sugar.

The experiments on the resting unnarcotized dog and those on the human beings have not been tabulated since they did not indicate significant changes in the lactic acid and glucose content of the blood.

Finally, a word should be said concerning the effect of the organs returning blood to the portal vein on the lactic acid and glucose content of the portal blood. Of thirty-nine observations on twelve amygalized and nine decerebrate dogs these organs added lactic acid fourteen times and removed it in eleven instances, while the results were not significant in fourteen observations. The findings in the case of glucose were more decisive. In twenty-nine experiments glucose was added only three times, while it was removed on sixteen occasions. The differences in ten instances were not significant.

DISCUSSION.

From these results it is apparent that the lactic acid liberated in the blood stream from muscle is removed by the liver, while the glucose added to the circulating fluid by the liver is absorbed by muscle. These two phenomena are not independent, but form a complete cycle since the lactic acid removed by the liver is converted to glycogen, and the glycogen of the liver may be broken down to enter the blood stream as glucose. This glucose may then be removed by muscle where it is synthesized to form glycogen, only again to be split to lactic acid.

Amygalized Dogs.—Any event which tends to raise the lactic acid content of muscle may cause an outpouring of lactic acid to the blood. Such a phenomenon may be produced by narcotics. Long (1928), Best, Hoet, and Marks (1926), and Graham (1929) have shown that narcotics may interfere with the recovery phase in muscle, though Hinsey and Davenport (1929) and Eadie (1929) have not been able to demonstrate a decrease of glycogen in the resting muscles of amygalized cats. The diminished ability of

muscles of narcotized animals to resynthesize glycogen from lactic acid is in accordance with our findings of a continuous outpouring of lactic acid. The level of the lactic acid of the arterial blood of the experimental animals, however, usually does not rise under amytal, probably because of the constant removal of the lactic acid by the liver. When this effect of the liver is eliminated by functional hepatectomy (Table IV) the lactic acid content of the arterial blood steadily increases.

Decerebrate Dogs.—To a certain extent changes observed in decerebration are similar to those occurring in narcosis. Perhaps due to a heightened extensor tonus, the muscles continuously liberate lactic acid. This tends to elevate further the high level of lactic acid produced during the 10 or 15 minutes of ether anesthesia necessary for the operative procedures. However, the liver here as under narcosis is able to reduce the concentration of lactic acid in the blood, as shown in Tables I, II, and V.

During exercise the muscles of the decerebrate and amyralized dogs usually released increased amounts of lactic acid, while the liver, as in the resting animals, continued to remove that substance from the blood.¹

Unnarcotized and Unoperated Resting Dogs.—It is difficult to demonstrate the liberation of lactic acid by muscle and its removal by the liver in the resting unnarcotized and unoperated animal. Janssen and Jost (1925) and the writers (Table III), confirming the findings of Barr and Himwich (1923) on human beings, find that the resting muscles of dogs remove lactic acid from the blood when that substance appears there in increasing concentrations. Janssen and Jost (1925) further noted that except when the concentration of lactic acid in the blood was suddenly augmented, resting muscles pour lactic acid into the blood stream. By careful control they avoided any of the possible effects of change of

¹ In the calculations of the effect of the liver on the concentration of lactic acid of the blood passing through it, Burton-Opitz's findings (1911) were used; namely, that three-tenths of the afferent blood comes in the hepatic artery and seven-tenths in the portal vein. In most of the present observations the hepatic vein contained less lactic acid and more glucose than either the portal vein or hepatic artery. Hence, irrespective of the exact partition of the afferent blood between hepatic artery and portal vein the liver would still remove lactic acid from the circulating fluid and add glucose to it.

temperature demonstrated by Bazett and Sribyatta (1928). Their dogs, although unnarcotized, were subjected to spinal cord transection, and therefore, were not unoperated animals.

The observations on the quietly resting human subjects and the unnarcotized dog yielded differences which in most instances were not beyond the experimental error. However, studies made on rabbits by Cori, Cori, and Goltz (1923) and on human subjects by Friedenson *et al.* (1928) disclose the removal of glucose by resting tissues. The experiments of Verzar (1912-13) and Krogh (1918-19) demonstrated a low oxygen tension in resting muscle. This indicated that there may be insufficient oxygen to reconvert to glycogen all the lactic acid formed in resting muscle. A statistical study might reveal, therefore, the liberation of lactic acid by muscle, a process accentuated by decerebration and narcosis.

Glycogen of Muscle and Liver.—There is a store of glycogen in muscle and liver which is built up in part from ingested carbohydrates as well as from the carbohydrate moiety of protein. Moreover, the relative amounts of glycogen in muscle and liver are influenced by oxidations as well as by the two antagonistic processes of the cycle. Cori and Cori (1928) have shown that adrenalin in the unnarcotized rat probably causes an outpouring of lactic acid from muscle, thus increasing the glycogen content of liver at the expense of that of muscle, although Eadie (1929) working on amytalized cats found a decrease in the glycogen of the liver after injection of adrenalin and practically no change in the muscle glycogen. Choi (1928) observed in decapitate cats that the glycogen content of muscle decreased only when it exhibited fibrillary twitchings as a result of the injections of adrenalin. Cori and Cori (1928) have also noted that insulin stimulates glycogenesis in muscle, presumably due to increased removal of glucose from the blood. In the present experiments on decerebrate dogs, the glucose gained by muscle is approximately balanced by the loss of lactic acid. Hence the small decrease of glycogen found by Hinsey and Davenport (1929) in muscles exhibiting extensor tonus must be imputed to oxidations. In the experiments on the amytalized dogs, the amount of glucose absorbed by the resting muscles was greater than the quantity of lactic acid yielded to the blood. Here the constancy of the

glycogen content of muscle found by Eadie (1929) and Hinsey and Davenport (1929) under amytal anesthesia must be explained by oxidations in muscle, while the decrease in glycogen observed by Long (1928) and Best, Hoet, and Marks (1926) must also be attributed to the same cause.

The liver, both in the amytalized and decerebrate fasted dogs, released more carbohydrates in the form of glucose than it received as lactic acid. Unless glycogenesis from sources other than lactic acid is sufficient to maintain both the output of glucose and its oxidation in the liver, a progressive decrease in the glycogen content of that organ might be expected. Eadie (1929) observed decreases in the glycogen content of the livers of amytalized cats.

Recovery in the Liver.—It is possible to evaluate the extent of the recovery occurring in the liver by taking into consideration the facts that three-tenths of the weight of our 20 kilo dogs is due to muscle (Schleier, 1923; Junkersdorf, 1925), and that the venous flow per gm. of muscle is 0.0015 cc. per second (Himwich and Castle, 1927). If the muscles of the dogs are liberating lactic acid at the rate of 10 mg. per cent, this indicates that all of the muscles of the body are liberating 3.2 gm. of lactic acid per hour. With the maintenance of a constant level of lactic acid in the blood, the liver was probably removing the greater part of the lactic acid escaping from muscle, although other organs were also involved in this process, as will be considered in subsequent communications.

It is interesting to note that the blood supply of the liver is sufficient to warrant the removal of large amounts of lactic acid. On the basis of the blood flow of the liver being 84 cc. per minute per 100 gm. of tissue (Burton-Opitz, 1911) and of our findings that the weight of the liver is between 2.5 and 3 per cent of the total weight of the experimental animals, it can be calculated that the total minute volume of the liver is somewhat less than 0.5 liter, while that of the total resting musculature of the body is a little larger than 0.5 liter.

During moderate exercise, when the muscles did not liberate more than 10 mg. per cent of lactic acid, the level of the lactic acid in the blood did not rise markedly. The exercise employed was of the same type and severity as that used in the experiments of Himwich and Rose (1929) when the rate of blood flow was 0.0048 cc. per second per gm. of muscle. If one-third of the muscles

of the body was involved in this work, 3.5 gm. of lactic acid per hour would be liberated, and if the remaining musculature added 2.2 gm., the total amount removed by the liver would be approximately 5.7 gm. per hour.

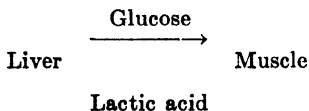
The relative amounts of recovery in liver and muscle may be compared both during rest and exercise. Although Elias and Schubert (1918), Janssen and Jost (1925), and Abramson, Eggleton, and Eggleton (1927) could discover no production of glycogen in muscle when lactic acid was injected directly into the blood stream of dogs, yet it is possible that synthesis of glycogen from lactic acid does take place in mammalian muscle, since we noted that muscles with exercise more moderate than that used in most of the present experiments added no lactic acid to the blood. Similar observations have been reported by Bornstein and Roese (1929). In the following calculations the observations of Meyerhof and Himwich (1924) were used: 1 cc. of oxygen causes a disappearance of from 1.6 to 2.7 mg. of lactic acid in excised mammalian (rat) muscle. With an approximate value of 2 mg. of lactic acid, and an oxygen consumption of 0.356 cc. per hour per gm. of muscle (Himwich and Castle, 1927), the total musculature of the resting body would be removing 4.3 gm. of lactic acid per hour, and since it is calculated above that the liver may remove 3.2 gm. of lactic acid, the muscles would be responsible for the disappearance of almost three-fifths of the lactic acid removed. With the degree of exercise occurring in these experiments Himwich and Rose (1929) observed a consumption of 2.22 cc. of oxygen per hour per gm. of tissue. With one-third of the musculature of the body exercising under these conditions 8.9 gm. of lactic acid would be removed within the muscles, and adding 2.8 gm., the recovery occurring in the resting muscles, the total is 11.7 gm. With the liver removing 5.7 gm., the muscles during moderate exercise would cause the disappearance of approximately two-thirds of all the lactic acid removed.

As exercise becomes progressively more severe the oxygen consumption of muscle rises to a maximum and the amount of lactic acid removed in muscle also increases. In contrast with muscle it is probable that the amount of lactic acid removed by liver may diminish, for with greater effort blood is shunted to the more active parts. It is therefore likely that the blood supply of the liver is decreased during severe exertion. However, this diminu-

tion of the afferent supply of the liver may be compensated to some degree by the removal of more lactic acid from each unit of blood as the concentration of the lactic acid in the circulating fluid increases. During recovery from exercise more blood should go to the liver and again it probably removes as large an amount of lactic acid as under rest or more moderate exercise. When exercise is severe and the muscles are pouring out 20 to 40 mg. per cent of lactic acid, the level of lactic acid in the blood rises rapidly, indicating that the liver, like muscle, is unable to cause the disappearance of more than limited amounts of this substance. Irrespective of the exact amounts of glycogen formed from lactic acid in muscle and liver, the fact of importance is that recovery does take place in the liver, since it has been demonstrated in the present work that muscle absorbs glucose from the blood and yields lactic acid to it, while the liver simultaneously removes lactic acid from the circulating fluid only to surrender carbohydrate in the form of glucose.

SUMMARY.

The glucose and lactic acid content of the blood of twenty-nine amyotized and eleven decerebrate dogs was studied during rest and exercise. As indicated in the schema, a glucose-lactic acid cycle exists between muscle and liver. Lactic acid liberated from muscle finds its way to the liver through the blood stream, and is there converted to carbohydrate. The liver in turn yields glucose to the blood, thus affording a supply of carbohydrate to muscle.



It must therefore become apparent that by virtue of this cycle, the liver becomes an integral part of the mechanism of recovery.

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CEREALS AND RICKETS.

III. THE COMPARATIVE RICKETS-PRODUCING PROPERTIES OF CORN, WHEAT, AND OATS, AND THE EFFECT OF IRRADIATION AND MINERAL SUPPLEMENTS.*

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Soon after the completion of our first experiments on the anti-rachitic activation of foods in 1923, we turned our attention to the activation of cereals. We were led to this, not because of the prominent part played by cereals in human and animal nutrition, but because we were particularly desirous of ascertaining what components of the ration and specifically what substances were capable of acting as acceptors for the ultra-violet energy, and because cereals happened to be the important constituent of the early rations used in our experiments (1).

In the various series of experiments which were run at that time, there were included certain groups on untreated cereals, with the technique used by McCollum *et al.* (2) in which rats of a suitable age and weight were fed on a very high intake; *viz.*, 3 per cent of the ration as calcium carbonate. The general conclusions drawn from this work, which have been got together only recently (3), were that untreated cereals as supplemented in these rations are decidedly rickets-producing. This was to be expected from the general reputation of cereals, obtained from practical feeding experience. What was new was the fact that ultra-violet radia-

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tion was efficacious in preventing the occurrence of the extremely rachitic condition.

At this time there were already in existence certain reports of Mellanby (4) pointing out that cereals do not produce rickets in dogs in proportion to their deficiency in calcium and phosphorus. This idea represented a decided departure from the older point of view because it was generally supposed that cereals were rickets-producing in proportion to their low content of bone-forming elements, or in proportion to the depressing effect of an excess of calcium on the availability of the phosphorus. The point which was emphasized by Mellanby was that of all cereals investigated, oatmeal was the worst offender, and white flour the least, with maize, barley, rye, whole wheat flour, and rice occupying intermediary positions. With the commonly accredited and emphasized deficiency of white patent flour in meeting the mineral requirements of animals, these claims were revolutionary, especially in light of the evidence on which they were formulated. We were particularly struck with this because in our experiments, limited though they were, our controls had given no indications of the inferiority of rolled oats compared with maize. However, in full realization of the possibilities of differences of opinion originating in results secured with different types of experiments, experiments differing not only in the kind of animals used, but also in the various dietary supplements fed with the cereal, we withheld comment until our experimental findings were more extensive. With the exception of a brief report given in 1927 (5), we have accordingly withheld publication until the present.

EXPERIMENTAL.

Experiments of 1924.—These experiments represent the preliminary trials which were referred to in the introduction. Young rats varying in age from 24 to 29 days and weighing from 60 to 74 gm. were placed on the McCollum Diet 3143 (2) without regard to litter origin or sex. In Diet 3143, consisting of wheat 33, corn 33, gelatin 14, wheat gluten 15, sodium chloride 1, and calcium carbonate 3, the cereal portions of wheat and corn were supplied entirely by one cereal in the respective experiments. The experimental rations were fed for a period of 5 weeks. The animals were then killed with ether, the femurs were dissected out, placed

in alcohol and extracted in a Soxhlet extractor for a number of days to effect complete removal of lipoids. They were then dried, weighed, and ashed. The distal ends of the long bones, particularly the radii and ulnæ were examined for rachitic involvement. This, as is well known, is indicated by a widening of the epiphyseal line to a wide rachitic metaphysis. The ribs were also examined grossly for enlargement of the costochondral junctions.

In general it can be said that all the long bones had very wide rachitic metaphyses and the costochondral junctions were decidedly enlarged. The extracted and dried femurs weighed the least for Shredded Wheat and patent flour rations, namely 106 and 107 mg. respectively; the most for wheat, corn-meal, Corn Flakes and rolled oats, with respective values of 127, 128, 121, and 122 mg. Cream of Wheat gave intermediate values of 112 and 110 mg.

The weight of ash in the femurs was highest for wheat at 41 mg., with a range from 29 mg. for corn-meal to 33 mg. for rolled oats and patent flour.

The percentage of ash in the femurs was highest for wheat and Shredded Wheat, namely 31.4 and 31.7 respectively, and lowest for corn-meal, 22.3 per cent. Patent flour gave 30.7 per cent of ash; Cream of Wheat, 27.6 per cent; Corn Flakes, 36.7 per cent; hominy, 27 per cent; and rolled oats, 26.8 per cent. It is seen that in general cereals are decidedly rickets-producing because femurs produced on these rations with the addition of vitamin D in the form of cod liver oil have an ash content of approximately 55 per cent.

Experiments of 1925.—These experiments in contrast with those of 1924 were projected for the distinct purpose of comparing corn, wheat, and oats for their rickets-producing properties. These cereals were incorporated in our Ration 2965 (6) consisting of cereals 76 parts, wheat gluten 20 parts, sodium chloride, 1 part, and calcium carbonate 3 parts. In order to determine to what extent calcification could be produced when an abundance of vitamin D was present, controls were run in which the entire rations containing the various cereals were exposed in thin layers for 30 minutes to the radiations of a quartz mercury vapor lamp.

Three litters of rats aged 27 and 28 days as raised on our stock ration were used for these experiments. One animal from each

litter was put on each one of the six rations, giving a litter representative for each cereal. Their initial weights ranged from 55 to 75 gm. They were kept on the experimental rations for 5 weeks,

TABLE 1.

Comparative Calcifying Properties of Cereals in Rat Experiments of 1925 As Shown by Analysis of Femurs.

Cereal.	Rat No.	Weight of femur. gm.	Weight of ash. gm.	Ash. per cent
Yellow corn.	1	0 1115	0.0260	23 3
	2	0 1120	0 0260	23 2
	3	0 1104	0 0250	22 6
		0 1113	0.0257	23 0
Rolled oats.	4	0 1028	0 0324	31 5
	5	0 1284	0 0374	29 1
	6	0 1352	0 0368	27 2
		0 1221	0 0355	29 3
Wheat.	7	0 1384	0 0528	38 1
	8	0 1563	0 0605	32 5
	9	0 1492	0 0484	38 7
		0 1478	0 0525	36 4
Irradiated yellow corn.	10	0 1342	0 0624	46 5
	11	0 1812	0 0880	48 5
	12	0 1592	0 0704	44 2
		0 1582	0 0736	46 4
Irradiated rolled oats.	13	0 1582	0 0798	50 0
	14	0 1930	0 0832	43 1
	15	0 1854	0 0850	45 8
		0 1789	0 0826	46 3
Irradiated wheat.	16	0 1812	0.0933	51.4
	17	0 2948	0 1464	49.6
	18	0 2418	0 1240	51.2
		0 2393	0 1212	50 7

at the end of which time they were killed and tissues removed for examination as before.

At the end of the 5 week period the animals on the corn ration weighed 88 to 100 gm., on the oat ration 66 to 108 gm., on the

wheat ration 100 to 103 gm., on the irradiated corn ration 73 to 120 gm., on the irradiated rolled oats 83 to 99 gm., and on the irradiated wheat ration 98 to 145 gm. These figures revealed that in general the growth on the non-irradiated cereals was practically the same for all; on the irradiated ration, however, growth on the wheat ration was much superior. The records of food consumption were not satisfactory, due to wastage of ration. This was especially true in the case of the animals on the irradiated cereals, so that little reliance could be placed upon them. One factor which had not been given sufficient attention was the degree of comminution of the grains. They had been ground in an Excelsior mill to a fine powder but this was not uniform for the different cereals.

The results on the production of bone specifically with respect to weights, ash content, and percentage of ash in femurs are shown in Table I. Yellow corn produced the lightest bone containing the least weight of ash, and also the lowest percentage of ash. Rolled oats was next in order and wheat the next. All of the cereals were definitely improved by irradiation because all the animals on the irradiated ration produced larger bones with a higher content of ash. The order of their efficiency in producing calcification was not changed from that of the non-irradiated cereals although the percentage of ash on yellow corn was equal to that produced by oats. Ophthalmia occurred in three of the oat-fed animals during the 4th and 5th weeks and was indicated in the 5th week in two of the wheat-fed animals. The part that this played in determining the results cannot be evaluated at this time.

The animals on the irradiated cereals were all very active up to the end of the experiment; from the non-irradiated cereal groups they were least active on corn and most active on wheat. These observations on behavior in general tally with what might have been expected from the ash analyses. Similarly the costochondral junctions of the corn-fed groups were enlarged the most, and the metaphyses of the distal ends of their radii and ulnæ were the widest; next in order were the oat-fed animals and the least abnormal were the wheat-fed animals.

Experiments of 1925-26.—The experiments of 1925-26 represented an elaboration of the series of 1925 just presented.

The cereals, corn, wheat, and oats, were fed in the irradiated and in the non-irradiated state, all with 20 per cent wheat gluten and 1 per cent sodium chloride, and with 0, 1, 2, and 3 per cent calcium carbonate for both the irradiated and the non-irradiated rations respectively. Various levels of calcium carbonate were used in order to make the comparisons of the rickets-producing properties of the three cereals more complete, the supposition being that any cereal less rickets-producing would require more of the carbonate to make it equally rachitogenic, because excess of calcium carbonate reduces the assimilation of the phosphorus of the ration. As it is well known that rickets is produced by low phosphorus as well as by low calcium assimilation, the number of experiments was increased to include a duplicate set in which the phosphorus intake was equalized. This was done, after analyses of the different cereals had been made, by the addition of phosphoric acid to the rolled oats and corn rations. 1.817 gm. of phosphorus as H_3PO_4 per kilo of ration were added to the corn ration and 0.717 gm. to the oat ration. The rations in this series were finely ground in a pebble mill.

168 piebald rats raised in our stock colony were used for these experiments. Their ages ranged from 24 to 30 days and their weights from 48 to 70 gm.—specifically 17.8 per cent were 24 days old, 27.9 per cent 25 days, 37.5 per cent 26 days, 12.5 per cent 27 days, 0.6 per cent 29 days, and 3.5 per cent 30 days. 2.3 per cent weighed from 48 to 50 gm., 17.86 per cent from 51 to 55 gm., 38.6 per cent from 56 to 60 gm., 28.5 per cent from 61 to 65 gm., 11.9 per cent from 66 to 70 gm., and 0.6 per cent 70 gm. Of the forty-two experimental groups, three contained three males and one female; three contained one male and three females, and the others contained two males and two females. Hammett (7) showed that female skeletons contain a slightly higher percentage of ash.

The rats were kept in groups of four in our wire screen cages provided with false bottoms. The rations were fed *ad libitum*. Attempts were made to obtain consumption records but the desirable degree of accuracy was not attained, due to variation in spillage. In a limited number of experiments the rats were segregated in individual cages and given weighed portions of ration daily, the residues being weighed back each day. A record with

one group taken for a period of 23 days showed that the average consumption per animal was 7.2, 5.5, and 7.4 gm. respectively for corn, oats, and wheat, and 8.3, 5.6, and 8 gm. for the same cereals irradiated. In all cases the consumption of rolled oats was the lowest and irradiation increased the consumption for all the cereals although not uniformly. At the end of 5 weeks the rats were killed with ether and tissues were removed for examination as described before.

Observations on the animals showed that for optimum results the rations used by us were dangerously low in vitamin A. It was observed by Steenbock and Coward (8) that a sample of wheat contained more vitamin A than oats or white corn. In the present

TABLE II.

Increases in Weight of Rats on Cereal Rations without Phosphorus Addition and with Phosphorus Content Equalized.

Experiments of 1925-26. Figures are averages of four animals.

CaCO ₃ , per cent.....	Without P addition.				P content equalized.			
	0	1	2	3	0	1	2	3
	gm	gm	gm.	gm.	gm.	gm	gm.	gm.
Yellow corn.	10	52	47	38	18	38*	35	37
Whole wheat.	15	41	41	44	11	37	39	28
Rolled oats.	7	33	41	36	2	21	23	24
Yellow corn (irradiated).	56	57	58	33	56	53	48	47
Whole wheat (irradiated).	55	45	41	41	50	54	29	44*
Rolled oats (irradiated).	40	30	28	29	38	28	43	39

* Average of three rats.

experiments the superiority of yellow corn discovered by us (9) in 1918 was again demonstrated because in the sixteen groups with four animals on each cereal, ophthalmia occurred in only one corn-fed group during the last week of the experiment. On the other hand, ophthalmia was observed in six wheat-fed groups and in eleven oat-fed groups sometimes in the 4th week, but mostly in the last week of the experiment. It must, however, be noted that the ophthalmia was mostly of Stage 1, characterized in severity by slight accumulation of exudate and swelling, with rarely an incipient erythema, and never any purulency. As yellow corn was used, the rare occurrence of ophthalmia in the corn-fed groups was to be expected.

We do not know whether or not a deficiency of vitamin A in our rations affected our data on calcification. Correlating the weights of the animals obtained weekly with the incidence of ophthalmia revealed no indications of the ophthalmia having interfered with growth. With only a very few exceptions the animals continued their established rate of growth or maintenance after the first 2 weeks, without a let-up to the end of the 5 week period. This was the general case even when the ophthalmia was indicated during the 3rd week.

TABLE III.

Weights of Femurs of Rats on Cereal Rations without Phosphorus Addition and with Phosphorus Content Equalized.

Experiments of 1925-26. Figures are averages of four animals.

CaCO ₃ , per cent. . . .	Without P addition.				P content equalized.			
	0	1	2	3	0	1	2	3
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Yellow corn.....	0.0904*	0.1579	0.1227	0.1070	0.0929	0.1034†	0.1688	0.1324
Whole wheat.....	0.1004	0.1824	0.1705	0.1552	0.1003	0.1463	0.1455	0.1444
Rolled oats.. . .	0.0948	0.1695	0.1455	0.1215	0.0968	0.1276	0.1479	0.1376
Yellow corn (irradiated).....	0.1159	0.1730	0.1747	0.1383	0.1069	0.1889	0.2181	0.2167
Whole wheat (irradiated)....	0.1212	0.2166	0.2071	0.1745	0.1235	0.1930	0.1915	0.2014†
Rolled oats (irradiated).....	0.1222†	0.1898	0.1868	0.1462	0.1035	0.1472	0.1938	0.1959

* Average of two rats.

† Average of three rats.

In Table II are given the average increases in weight of the animals for all groups. In general, growth on rolled oats was less than on wheat and corn. This can be understood in view of the fact, already related, that consumption of that ration was less than of the others. The primary deficiency of the cereals for growth was evidently calcium because when calcium carbonate was added up to 1 per cent of the ration, growth increased on all the cereals. Larger amounts of calcium carbonate did not bring about proportionate increases. In fact in some instances growth was actually decreased but no generalization for any one cereal can be made. The addition of phosphorus to equalize the phosphorus intake did

not change the picture materially. But an outstanding result was the pronounced increase in weight brought about by irradiation. Growth was increased by irradiation to the maximum extent observed in the series, even when no calcium carbonate was added. In fact, raising the calcium carbonate content in the six

TABLE IV.

Ash in Femurs of Rats on Cereal Rations without Phosphorus Addition and with Phosphorus Content Equalized.

Experiments of 1925-26. Figures are averages of four animals.

CaCO ₃ , per cent.....	0		1		2		3	
A. Without P addition.								
	gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent
Yellow corn.	0 0393*	43.8*	0 0683	43 2	0 0373	31.3	0 0316	29 4
Whole wheat.	0 0427	42 3	0.0942	51 5	0 0801	46 6	0 0680	43 6
Rolled oats.. . . .	0 0414	43 7	0 0840	49 5	0 0583	39 8	0 0430	35.2
Yellow corn (irradiated).. . . .	0 0472	40 8	0.1193	54 5	0 1015	49 4	0.0649	47.0
Whole wheat (irradiated)... . .	0 0536	44 3	0.1225	56 9	0.1098	53 2	0.0925	52.9
Rolled oats (irradiated)	0 0518*	42 3*	0.1028	53 9	0 0940	50 2	0.0727	49.9
B. P content equalized.								
Yellow corn.	0.0343	36 8	0.0724*	52.3*	0.0920	54 4	0.0566	44.4
Whole wheat.	0 0396	39 5	0.0753	51.6	0 0693	47.8	0 0545	42 2
Rolled oats.	0.0387	40.3	0 0673	52.6	0.0742	50.8	0.0558	42.7
Yellow corn (irradiated).	0 0416	38.9	0.1077	57.0	0.1286	58 8	0 1176	54.2
Whole wheat (irradiated).. . . .	0 0504	40 9	0.1076	55.8	0.1041	54.3	0.1058*	52.3*
Rolled oats (irradiated).....	0 0419	40 6	0 0788	53 6	0 1091	56 2	0 1054	53.7

* Average of three rats.

irradiated groups even to 1 per cent gave instead a total decrease in weight of 33 gm. in four of the groups and a slight increase of 5 gm. in the remaining groups.

The weights of the femurs are given in Table III. These are out of line with the increases in body weight as shown in Table II.

Unsupplemented untreated rolled oats at all levels of carbonate intake produced heavier bone than yellow corn. Wheat, however, produced the heaviest bone. When irradiated, calcification was much improved in all cases, but the order of efficiency of the cereals in general was not changed. Further modification of the rations, namely equalizing the phosphorus content, did not bring the cereals to a parity; wheat in general was still superior. Rolled oats in general produced the heaviest bones and corn the poorest. However, when the cereals were irradiated, corn in two instances produced even heavier bone than wheat.

The weights of ash in the femurs are shown in Table IV, A. In general, the ash weights follow the bone weights in order. On the unsupplemented cereals, untreated and irradiated wheat gave the highest values, with the oat lots next and the corn lots last. When the phosphorus content of the rations was equalized, the weights of bone ash still differed. Correlations are then made with difficulty. It may, however, be said that without additions, wheat gave the best results, oats the next best, and corn the poorest. But with the carbonate additions, corn in a few instances gave even more bone ash than wheat.

The percentages of ash in the femurs as calculated are presented in Table IV, B. When no phosphorus additions were made, it will be seen that in general wheat produced the highest values, rolled oats the next highest, and corn the lowest. With phosphorus additions oats in general gave higher values than either wheat or corn, the latter two being almost brought to a parity.

From the foregoing it may be concluded that the wheat kernel is capable of producing not only heavier bone but bone of higher ash content, and a higher percentage of ash, and therefore in all respects better bone than corn or oats. Of the two latter, rolled oats showed itself to be superior in producing heavier and better bone than corn.

Furthermore it is clear that irradiation of the cereals increased growth on all the cereals even when not supplemented with calcium carbonate. Inasmuch as all cereals are very low in their calcium content, and inasmuch as the calcium requirements of animals are relatively large, this effect on first thought is rather surprising. It apparently demonstrated what might have been expected, that vitamin D as produced by irradiation also brings

about a stimulation in other ways than in the assimilation of calcium. However, while the irradiated cereals produced heavier bone, the limitations in the supply of calcium were nevertheless operative in preventing an increase in the percentage of ash in these bones.

The addition of calcium carbonate to a certain extent produced the same effect as irradiation, provided the calcium carbonate was not increased above 1 per cent. Up to 1 per cent with wheat and oats it increased the weight of the bone, the weight of the bone ash, and the percentage of ash contained in the bone. When the cereals were irradiated in addition to being supplemented with 1 per cent calcium carbonate, still further improvement in bone formation took place. The depressing effect of an excess of calcium in the ration was again demonstrated because when the carbonate was increased above 1 per cent of the ration, all three of the aforementioned desiderata failed to be accomplished. When the cereals were irradiated, the decreases brought about by the excessive carbonate additions were moderated. In view of the depressing effect of excessive carbonate administration, it might be surmised that the difference in the calcifying properties of the different cereals lay in the difference in their phosphorus content, but apparently our data do not support this possibility. Equalization of the phosphorus content of the cereal rations did not make them equally effective in bone formation, but it did make the corn more efficient with its calcium carbonate supplement. It must, however, be considered that inorganic phosphorus added as phosphoric acid may not be equivalent in physiological properties to the organic phosphorus compounds in the cereals. Our experiments certainly demonstrate the fact that inequality of phosphorus intake is not the sole reason for the difference in calcifying action of various cereals.

Experiments of 1926-27.—It was realized that a critical difficulty of the preceding experiments lay in the difference in the amounts of the various rations consumed. Much has been said of the value of keeping consumption records (10), but little has been done in the way of interpreting results even with these available. When the consumption of rations is found uniform, difference in performance of the animals need not of course be qualified in relation to anything but the quality of the ration, but when the

consumption is not the same for the different groups and the performance of the animals is likewise different, it is impossible to conclude whether the difference in performance is due to variation in consumption or to other factors. It, therefore, seemed to us that the simplest way of eliminating variations would be to equalize the intake, limiting all animals of the various groups in a series to the smallest amount voluntarily consumed by any member of the series. It stands to reason that the individual of reference must of necessity not be abnormal.

It is true that equalizing the consumption may also introduce certain inconsistencies of experimentation, because of the fact that some individuals are possessed by heredity of greater growth impulse than others. This growth impulse might lead to the production of larger bone as indicated by weight which, due to limitation of mineral intake, would result in a lower percentage of ash contained therein. However, when this obtains, the total ash content of such bone should still be larger than when the growth impetus was of a lower order. By making correlations of bone weight, weight of ash contained therein, and percentage of ash it is believed that differences brought about by variations of growth impulse will not remain unrecognized and suitable correction can be made.

It is, however, assumed by this system of evaluation that the various factors operative in bone production act independently and consistently without one modifying the influence of the other. This assumption, in view of the fact that we (11) have found vitamin D to be necessary for the growth under certain circumstances—which has been confirmed by others (12)—probably is not always justified but we are taking the liberty of assuming its justifiability for the moment. It is expected that whatever errors may result with this system, will later be possible of analysis and in no case can the data be as involved as they have been in experiments carried out heretofore.

With the above relations in mind, 144 piebald rats raised in our stock colony were put on the various cereal rations at ages varying from 22 to 28 days. They weighed from 55 to 70 gm. 4.1 per cent of the total was started at 22 days, 15.0 at 23, 22.9 at 24, 24.3 at 25, 15.2 at 26, 13.1 at 27, and 4.1 per cent at 28 days. 56.2 per cent weighed from 55 to 60 gm., 43 per cent from 61 to 65 gm.,

and 60 per cent from 66 to 70 gm. The sexes were fairly uniformly distributed among the groups; all except nine contained three males and three females. In the nine groups the relation of the sexes was 2:4, either males or females. They were kept in individual cages measuring $10 \times 10 \times 20$ inches. These cages were provided with screen bottoms measuring three meshes to the inch. Corn, wheat, and oats were the cereals fed. Each was ground to an impalpable powder and fed with wheat gluten and sodium chloride and calcium carbonate in our Ration 2965, with modifications. The corn used was white corn in order to bring the corn-fed groups as near to a parity with the wheat- and oat-fed groups with respect to content of vitamin A as possible. The cereals were analyzed for calcium and phosphorus by the McCrudden (13) and the phosphomolybdate magnesia precipitation (14) methods respectively. They were found to contain 0.010 per cent calcium for corn, 0.067 per cent for wheat, 0.097 per cent for oats; 0.54 per cent phosphorus for corn, 0.92 per cent for wheat, and 0.94 per cent for oats. The wheat gluten contained 0.101 per cent calcium and 0.70 per cent phosphorus. As rolled oats in this instance was slightly higher in phosphorus than wheat and corn, the phosphorus content of the wheat and corn rations was brought up in a few instances to that of oats by the addition of a solution of phosphoric acid. This was evaporated on the wheat gluten component of the rations in calculated amounts and finally checked up by analysis of the mixture. One series of the four was fed without calcium carbonate additions; the other with 3 per cent. Some of the rations were irradiated and others not. The general organization of the various experiments can be seen from Tables V to VII. All experiments were run for a period of 5 weeks. The animals were weighed weekly and finally killed for the removal of the different bone samples mentioned in the previous experiments.

In Table V are shown the actual data on feed consumption control, giving the ranges in individual consumption as well as the averages for all rats of a group. For the most part the rats were given initially 4 gm. of the ration. Daily consumption increased slightly, but towards the close of the period it fell off again so that the individuals consumed only about 1 gm. more than initially. In these experiments it was again our experience that rolled oats was not consumed so readily nor in such quantity as the other

TABLE V
Daily Consumption and Increase in Weight of Rats, Experiments of 1926-27.*

Series No	Daily consumption								Increase in weight.			
	1		2		3		4		1	2	3	4
	0		3		3		3		0	3	3	3
	Range.		Average.		Range.		Average.		Range.		Average.	
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
White corn.....	4 8-5 1	5 0	5 3-5 4	5 3	5 9-6 4	6 3			5	0 6	16	
Whole wheat.	4 9-5 3	5 1	5 3-5 4	5 3	6 4-6 4	6 4			4	6	23	
Rolled oats.	4 1-5 0	4 6	5 2-5 3	5 2	5 8-6 4	6 3	6 3-6 4	6 4	4	8	30	27
White corn (+ P ₂ O ₅).....	4 9-5 3	5 0	5 3-5 3	5 3	6 0-6 4	6 3			7	2†	20	
Whole wheat (+ ").....	5 1-5 3	5 2	5 3-5 3	5 3	6 4-6 4	6 4	6 2-6 4	6 3	5	4	25	14
White corn (irradiated).....							6 3-6 4	6 4				23
Whole wheat (").....							6 1-6 4	6 3	28	11	24	25
Rolled oats (").....							6 2-6 4	6 4				22
White corn (+ P ₂ O ₅) (irradiated).....							6 4-6 4	6 4				23
Whole wheat (+ ") (").....												

* Average of six rats.

† Average of five rats.

cereals. It appeared to us that the failure to consume oats readily did not result from discrimination due to lack of palatability, because during the 1st week the oat ration was consumed readily. It, however, is possible that the rats were not fed a sufficiently large quantity to test this out accurately, but the first portions given every morning were also consumed readily. We are at present unable to explain this difficulty of consumption of rolled oats. But the facts are, that sooner or later, some or all the rats on oats consumed less than the others.

There is a possibility that the difference in consumption was due to physical factors. We found that the rolled oats ration was more bulky; that is, took up more space, gram for gram, in the feeding cup than the corn or wheat rations. It was also true that the rolled oats as ground in our Excelsior mill was of such a physical nature that it adhered to the sides of our ball mill, thereby preventing further comminution. It was not considered advisable to dry the oats with the aid of heat, although this would have prevented its adhesion, because this might have changed its nutritive properties. We are quite certain that the difference in consumption was not due to the presence of very small and therefore scarcely visible particles of fiber in the oats, yet this possibility must be kept in mind.

Incompleteness of digestibility as a factor was ruled out by the following experiment: Twelve male rats weighing from 264 to 304 gm. were divided into three groups of four each. One group was given our Ration 2965, containing oats as the cereal; another contained corn and a third wheat. After they had been accustomed to their diets by preliminary feeding periods, feces were collected from each group daily for approximately 1 week each. The animals were then changed to a different cereal, and feces were also collected after a suitable period of adjustment to the ration. Later the animals were changed to the third cereal and collections made in the same manner. It should be stated that originally the animals were started on different cereals so that the sequence in the order of feeding the cereals was different. In all cases every rat was given 10 gm. of the ration daily. The feces were collected, dried, and weighed. The volume was determined by measuring in a graduated cylinder after covering the pellets with tobacco seed and then subtracting the volume of the seed from the mixture.

We found that the daily average weights of the feces were 1.11, 1.21, and 0.89 gm. for corn, wheat, and oats respectively, and the daily average volumes were 1.9, 2.3, and 1.4 cc. respectively. No difference was found between the different periods; that is, with respect to the sequence of feeding. In all cases wheat produced the bulkiest and heaviest feces, and oats the smallest in volume and the lightest in weight. This shows definitely that consumption of the oat ration was not limited by excessive bulkiness of the fecal residues. On the other hand, the possibility that the fecal residues should not have been large enough for normal alimentation and therefore maintenance of appetite, does not look acceptable to us on general principles. Yet on this we have no data.

To determine further whether physical factors played a rôle in consumption, we cooked finely ground rolled oats in a steam cooker after the addition of hydrochloric acid. To each kilo of oats there were added 72.9 cc. of 10 per cent hydrochloric acid and sufficient water to make a mixture of 2 liters volume. The heating was continued at 15 pounds pressure for 5 hours. The mixture was then neutralized with sodium carbonate and dried at 48.5° in an air current for 98 hours. This produced sodium chloride totaling 1 per cent of the weight of the ration when wheat gluten and calcium carbonate had been added in the usual proportions as found in our Ration 2965. This treatment did not increase consumption. As a matter of fact the rats on the treated oats consumed an average of 5.7 gm. daily in contrast with 6.3 gm. on the untreated cereal.

We also raised the question of the antineuritic vitamin B and the heat-stable vitamin G. Smith and Hendrick (15) pointed out that rats fed rolled oats at a level of 40 per cent had their growth improved by the addition of yeast. We obtained the same results with resultant increased consumption up to 7.4 gm. per rat per day by the addition of 6 per cent autoclaved yeast. Untreated yeast increased the consumption still more; namely, up to 8.1 gm. On the basis of this, it might be argued that rolled oats is not only primarily deficient in vitamin G but also deficient in vitamin B or some other factor or factors carried by yeast. However, before the cause of reduced consumption on rolled oats can be attributed to these factors, other possible supplementing values of yeast to the cereals necessary for proper alimentation must be eliminated.

That difference in content of other vitamins might be responsible suggested itself. It will be recalled that in our experiments of 1925-26 we observed ophthalmia as a symptom of vitamin A deficiency in some of the lots. This was again our experience in these trials; in the first series it was observed in one rat on wheat, four on rolled oats, five on white corn plus phosphorus, one on whole wheat plus phosphorus, and one on irradiated oats. In the second series it was observed in two animals on corn, one on wheat, and two on irradiated rolled oats; in the third series it was observed in five animals on corn, five on corn plus phosphorus, five on wheat plus phosphorus, and one on rolled oats. In the fourth series it was observed in three on irradiated corn, five on irradiated corn plus phosphorus, and one on irradiated wheat plus phosphorus. With one exception all the ophthalmia was of a very mild type. The exception occurred with four rats on rolled oats in Series 1, Table V. In general, ophthalmia occurred in all groups and even more on white corn than on oats. A deficiency in vitamin A therefore could not be held responsible for decreased consumption. Originally, we contemplated supplementing all our cereals with vitamin A, but this was not done because we did not want to complicate the relations with vitamin D additions as we did not have a source of vitamin A entirely free from vitamin D available. Usually the addition of vitamin D has a pronounced effect on consumption with rachitic animals, undoubtedly because with the healing of the lesions, the animals throw off their lethargy and become active with resultant great caloric requirement.

Our consumption records, Table V, show some variation in spite of our attempts at equalization. The variations recorded are, however, not of moment in influencing the outcome of our experiments because in no case do they exceed 10 per cent of the total quantity of ration consumed. This we believe is well within the range of other uncontrolled variables in experiments of this type.

In Series 3 and 4 it will be noted that the consumption of the rations irrespective of the kind of cereal exceeded that observed in Series 1 and 2. Probably as a result of this the growth of the animals in these series was also greater. Without carbonate additions the average increases in weight are remarkably uniform for the three untreated cereals. It should, however, be mentioned that three of the animals on rolled oats lost weight slightly. The

TABLE VI.
Data for Femurs of Rats.

Series No.	Extracted weight.				Ash weight.				Ash			
	1	2	3	4	1	2	3	4	1	2	3	4
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	per cent	per cent	per cent	per cent
CaCO ₃ per cent.	0	3	3	3	0	3	3	3	0	3	3	3
White corn.....	0 0993	0 1125	0 1049	0 1400	0 0411	0 0436	0 0339	0 0685	41 5	38 3	32 3	
Whole wheat.....	0 1017	0 1204	0 1256	0 1544	0 0419	0 0506	0 0423	0 0836	41 4	42 0	36 1	
Rolled oats.....	0 1045	0 1158	0 1182*	0 1153	0 0418	0 0451	0 0412*	0 0450	39 9	38 6	34 9*	39 0
White corn (+ P ₂ O ₅).....	0 1118	0 1208*	0 1231		0 0478	0 0586*	0 0493		42 7	48 7*	39 7	
Whole wheat (+ ").....	0 1122	0 1069	0 1197	0 1400	0 0452	0 0495	0 0442	0 0685	40 3	46 2	36 9	48 9
White corn (irradiated).....				0 1544				0 0836				51 5
Whole wheat (").....				0 1633	0 0547	0 0765	0 0812		40 8	54 4	50 3	51 8
Rolled oats (").....	0 1325	0 1405	0 1613	0 1715								53 8
White corn (+ P ₂ O ₅) (irradiated)....				0 1632				0 0919				51 8
Whole wheat (+ ") (")....								0 0838				51 8

* Averages of five rats.

differences in the averages, however, were made up by the others. One rat on corn plus phosphorus and two on whole wheat plus phosphorus also lost weight, undoubtedly due to vitamin A deficiency. Irradiation, as in the experiments of the preceding years, again increased growth remarkably. When calcium carbonate was added, increase in weight on corn was found to be slightly less than in the other groups. In Series 2, two rats on white corn, one on rolled oats, and one on white corn plus phosphorus lost a few gm. in weight. In the other series all the rats gained consistently. In general, equalizing the consumption of the rations for all the rats in each series evidently did not provide all the groups with the amount of ration which they would have consumed *ad libitum*. But by controlling the consumption we accomplished two purposes: we kept the intake of calcium and phosphorus, as well as unknown factors favoring and inhibiting mineralization of bone, constant to the amounts found in each cereal. On the basis of this we believe that our conclusions should carry more weight than those derived from our other experiments. The costochondral junctions showed differences in their degree of rachitic involvement with respect to the different series. When no calcium carbonate was added, they were practically normal with only slight beading, but with 3 per cent calcium carbonate there was moderate to severe rickets with many cases of angulation.

The metaphyses gave a somewhat more differentiating picture. Without calcium carbonate additions they were all normal, but with this addition their average width was greatest for corn, least for wheat, and intermediate for oats.

What we consider our most important data on the representative efficiency of corn, wheat, and oats are shown in Table VI. Table VII taken from our experience with Series 1 is added to give an idea of the detailed observations obtained with individual, representative animals.

In general these details do not show the cereals to have decisive differences. In Series 1, where no calcium was added, oats was superior in that it produced the heaviest bone. However, oats was on a parity with wheat and corn for total ash and slightly lower than wheat and corn in percentage of ash.

In Series 2 and 3, where 3 per cent calcium carbonate was added, oats stood second to wheat in all respects. Corn was always

TABLE VII.
Detailed Data for Rats of Series 1, Table VI.

Ration.	Rat No.	Daily consumption.	Body weight.		Maximum gain.	Femurs.		
			Initial.	Final.		Extracted weight.	Ash.	
		gm.	gm.	gm.	gm.	gm.	gm.	per cent
White corn.	73	4 8	58	62	6	0 0984	0 0394	40 0
	74	5 6	64	67	8	0 0992	0 0437	44 1
	75	5 1	55	62	11	0 0959	0 0387	40 4
	76	5 0	61	64	8	0 0873	0 0407	46 6
	77	5 0	62	64	4	0 1088	0 0420	38 6
	78	5 0	56	63	12	0 1064	0 0418	39 3
Average.		5 0	59 3	63 7	8 2	0 0993	0 0411	41 5
Whole wheat.	79	5 1	63	68	6	0 1131	0 0492	43 5
	80	5 1	62	66	9	0 0958	0 0410	42 8
	81	5 3	60	67	12	0 1124	0 0494	44 0
	82	5 3	56	72	18	0 0974	0 0394	40 5
	83	5 1	62	62	3	0 0954	0 0344	36 1
	84	4 9	56	57	5	0 0963	0 0382	39 7
Average.		5 1	59 8	65 3	8 8	0 1017	0 0419	41 1
Rolled oats.	85	4 1	62	57	8	0 1011	0 0420	41 5
	86	4 5	61	58	3	0 1034	0 0426	41 2
	87	4 9	58	67	9	0 1194	0 0448	37 5
	88	5 0	65	76	11	0 1203	0 0504	41 9
	89	4 2	58	55	2	0 0825	0 0310	37 6
	90	4 7	57	71	14	0 1001	0 0399	39 9
Average.		4 6	60 2	64 0	7 8	0 1045	0 0418	39 9
White corn (modified).	91	4 9	61	74	13	0 1165	0 0515	44 2
	92	5 0	62	62	1	0 0995	0 0436	43 8
	93	5 3	57	71	14	0 1070	0 0483	45 1
	94	4 6	56	51	4	0 1022	0 0391	38 3
	95	5 3	60	69	12	0 1302	0 0532	40 9
	96	5 0	58	61	3	0 1151	0 0508	44 1
Average.		5 0	59 0	64 7	7 8	0 1118	0 0478	42 7
Whole wheat (modified).	97	5 2	57	67	10	0 1058	0 0443	41 9
	98	5 1	64	65	5	0 1134	0 0440	38 8
	99	5 0	58	55	3	0 0951	0 0418	44 0
	100	5 2	61	63	4	0 1066	0 0395	37 1
	101	5 2	58	70	14	0 1141	0 0445	39 0
	102	5 3	65	70	9	0 1382	0 0569	41 2
Average.		5 2	60 5	65 0	7 5	0 1122	0 0452	40 3

TABLE VII—*Concluded.*

Ration.	Rat No.	Daily consumption.	Body weight.		Maximum gain.	Femurs.		
			Initial.	Final.		Extracted weight.	Ash.	
		gm.	gm.	gm.	gm.	gm.	gm.	per cent
Rolled oats (irradiated).	103	5 2	59	86	27	0.1237	0 0502	40.6
	104	5 3	63	86	26	0 1304	0 0550	40.3
	105	5.2	63	95	32	0 1338	0.0515	38.5
	106	5 1	57	80	27	0.1182	0 0468	39 6
	107	5 3	55	92	37	0 1385	0.0576	41.6
	108	5.3	62	87	25	0 1443	0 0633	43 9
Average.		5.2	59 8	87.7	29.0	0.1325	0.0541	40 8

slightly inferior to both. Addition of phosphorus without calcium carbonate made corn and wheat produce heavier bone which also contained more ash, but this increase in ash did not keep pace with the total bone weight, with the result that the percentage of ash was not increased. When calcium as well as phosphorus was added to corn, the bone weight, ash weight, and percentage of ash were increased. With wheat the results were not consistent. It is, however, interesting to note that phosphorus additions increased the amount of ash in most cases even in those instances where phosphorus assimilation had not been depressed by calcium additions. Our results show very clearly that irradiation of the cereals tends to correct all difficulties within the limits of these experiments because it was always very effective in increasing weight of bone and weight of ash. The percentage of ash, however, was not increased unless calcium was added because the increases in dry weight and weight of ash were parallel and the lack of lime exercised a final limitation to further improvement in spite of the availability of excessive amounts of vitamin D.

SUMMARY.

The rickets-producing properties of corn, wheat, and rolled oats have been compared under various dietary régimes in which a total of 230 standardized rats was employed. These have been fed the various cereals, unsupplemented, supplemented with calcium carbonate, supplemented with phosphoric acid to equalize the total intake of phosphorus, and treated with the radiations of quartz mercury vapor lamps.

In general, regardless of the calcium carbonate or phosphoric acid additions, the cereals ranked in antirachitic potency in the descending order of wheat, rolled oats, corn. With calcium carbonate supplementation, the addition of 1 per cent calcium carbonate produced the best bone. Small amounts of added phosphoric acid effected no appreciable change in bone composition. Irradiation corrected the difficulties in calcification encountered and made the cereals approximately alike in antirachitic potency. Irradiated cereals produced heavier bone than the non-irradiated, but without calcium supplements the percentage of ash was not increased.

Data are also presented which may account for the comparatively poor consumption of rolled oats by rats. The possible rôle of a vitamin A deficiency and indigestibility has been practically ruled out. Hydrochloric acid hydrolysis also effected no improvement. On the other hand, the data suggest that a deficiency of vitamin G and to a lesser extent vitamin B, or some other factor resident in yeast, caused the eventual decrease in food intake.

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NOMOGRAM DERIVING BASAL METABOLISM FROM HEIGHT-WEIGHT COORDINATES.

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The normal basal metabolism per day is estimated by the method of Du Bois as the surface area of the body, calculated from the equation, $A = 0.007184 W^{0.425} H^{0.725}$ in which A represents area in sq. m., W weight in kilos, and H height in cm. (1) multiplied by the standard value of calories per sq. m. of body surface per hour for the sex and age group (2) (Table I), multiplied by 24.

The polyphase alignment chart of Boothby and Sandiford (3) makes this calculation in two stages. A straight edge laid between the proper points on the scales of height and weight intersects the scale of surface area. Readjusted to lie between this point and the proper point on the scale of sex and age group, it indicates the answer on the scale of calories. To this end two points must be spotted, a straight edge placed, an intersection marked, a fourth point spotted, the straight edge rotated, a value read. The manipulations are many and exacting.

All that is necessary to obtain the result in one step is to spot two coordinates, sight their intersection, and read off its value. The height-weight formula chart of surface area (1) can be modified to represent calories per unit of time for a uniform metabolic rate (4). By using logarithmic in place of numerical values and a separate scale for one of the variables in the formula for each metabolic rate, the calories per unit of time can be represented coincidentally for all metabolic rates. x and y axes are marked off as equidivisional scales of logarithms. A scale of height in inches, h , is plotted on the y axis according to the value each number receives when developed according to the expression, $0.725 \log 2.54 h$. A scale of weight in pounds, w , is plotted on the

x axis for each rate of calories per sq. m. per hour, c , according to the value each number receives when developed according to the expression, $0.425 \log 0.4536 w + \log 0.007184 + \log c + \log 24$. Graphs are drawn for a series of equations, $\log C = x + y$, in which C represents calories per 24 hours, and x and y the logarithms of the respective axes.

Chart 1 estimates the basal metabolism per day from sex, age, height, and weight. Go to the upper tier of weight scales for females, the lower for males, identify the scale for the required age group, spot the weight in pounds on it, trace an imaginary line per-

TABLE I.
Calories per Sq. M. of Body Surface per Hour (Height-Weight Formula).

Age.	Males.	Females.
<i> yrs.</i>	<i> calories</i>	<i> calories</i>
14-16	46.0	43.0
16-18	43.0	40.0
18-20	41.0	38.0
20-30	39.5	37.0
30-40	39.5	36.5
40-50	38.5	36.0
50-60	37.5	35.0
60-70	36.5	34.0
70-80	35.5	33.0

pendicularly into the field of the chart, spot the height in feet and inches on the nearest scale, trace an imaginary line horizontally to its intersection with the vertical line previously traced, read the number of calories from the nearest graph, or approximate more closely by interpolation.

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ANIMAL CALORIMETRY.

THIRTY-NINTH PAPER.

SPECIFIC DYNAMIC ACTION IN THE NORMAL AND PHLORHIZINIZED DOG.*

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It has been erroneously stated that this laboratory is of the opinion that all the amino acids which are convertible into glucose manifest specific dynamic action. It appears to be well established that glutamic acid may be converted into glucose to the extent of 3 of its 5 carbon atoms (1) and it is a fact that, in this laboratory at least, its ingestion has never been followed by any manifestation of specific dynamic action (2). We regret that our results differ from those of Grafe (3) and of Rapport and Beard (4).

If we accept our results as correct two questions arise: (1) May glutamic acid be oxidized without a production of sugar in the normal dog and hence give no specific dynamic action, but (2) may it not manifest specific dynamic action in the phlorhizinized dog because of its conversion into glucose? Another question of interest is, does protein, when given to a normal dog, cause the same specific dynamic action as it does when administered in the diabetic condition? In other words, does its certain transformation into large quantities of glucose in the diabetic state in any way modify its specific dynamic action?

It will be recalled that the specific dynamic action of glycine is the same in the normal as in the diabetic dog (5). It has also been established that the specific dynamic action of glycine is proportional to the quantity of the substance ingested (5). To this

* A paper presented before The Thirteenth International Physiological Congress held at Boston, August 19-23, 1929.

may be added the statement that *in the case of glycine the amount of extra calories of metabolism above the level of the basal metabolism (which increase is the measure of the specific dynamic action) is independent of the size of the dog.* For the increase in metabolism in terms of calories after administering 10 gm. of glycine is exactly the same in dogs weighing respectively 6.6 and 12.7 kilos. These relations appear in Table I.

The constancy of the reacting mechanism of the normally nourished dog to the ingestion of foods and their cleavage products should be sharply borne in mind.

TABLE I.
Increases in Calories Produced per Hour after Administration of Glycine.

	20 gm glycine.		10 gm glycine.			
	cal.	cal.	cal.	cal.	cal.	cal.
2nd hr.	9.6	7 0	4 8	4 6	4 0	4.5
3rd "	5 0	6 8	2 8	4 2	3 5	4.6
4th "	3.7	6 0	2.9	3 1	3.4	1.7
	18 3	19.8	10 5	11 9	10 9	10.8
Weight of dog, kg.....	12 7*	12.7*	12.7*	12 7*	6 6†	6.6†

In the experiment shown in the last column on the right the material administered was glycylglycine in amount corresponding to the equivalent of 9.75 gm. of glycine.

* Cf. (5).

† Plummer, N. H., Deuel, H. J., Jr., and Lusk, G., *J. Biol. Chem.*, **69**, 339 (1926).

Calculation Factors.

By calculating the values of the respiratory exchange and the heat produced from protein under varying D:N ratios in diabetes, and by calculating similar factors when glutamic acid is converted into glucose, one obtains the following values which have been used in the computations made in this paper (Table II). Glucose (D) has been computed as containing 3.755 calories per gm. The calculations are similar in nature to those already published from this laboratory ((2) p. 158, (5) p. 600, (6)).

Computation of Protein Metabolism.—It was found with Dog 61 that the average elimination of urinary N per hour during four

periods when the basal metabolism was determined was 0.154 gm. (maximum 0.169; minimum 0.133). When lard, 10 gm., was given with 1 gm. of Liebig's extract the urinary N per hour averaged 0.137 gm. in two periods (maximum 0.166; minimum 0.107). In the first instance 0.019 gm. was deducted in computing the total protein metabolism because of the average hourly increase in urinary N following the ingestion of Liebig's extract previously obtained in this laboratory (2). When glutamic acid, 20 gm., was given with lard, 10 gm., and Liebig's extract, 1 gm., the urinary

TABLE II.
Protein and Glutamic Acid in Diabetes.

	N-O ₂	N-CO ₂	R.Q.	N-calories.
	gm.	gm.		
Protein in diabetes (D:N = 3.65).				
Normal exchange for 1 gm. urine N.	8.49	9.35	0.802	26.51
Deduct for 3.65 gm. unoxidized D.	3.89	5.35		13.71
Exchange for 1 gm. urine N	4.60	4.00	0.632	12.80
D:N = 3.00.				
Exchange for 1 gm. urine N.....	5.29	4.95	0.680	15.24
D:N = 2.80.				
Exchange for 1 gm. urine N.....	5.50	5.24	0.693	16.00
Glutamic acid in diabetes (D:N = 7.25).				
Normal exchange for 1 gm. glutamic acid N.....	10.32	14.18	1.00	33.05
Deduct for 7.25 gm. unoxidized D.	7.73	10.63		27.22
Diabetic exchange for 1 gm. glutamic acid N.....	2.59	3.55	1.00	5.83

N in four experiments averaged 0.240 gm. per hour (maximum 0.273; minimum 0.185). The computation of the protein metabolism when glutamic acid was given was based on the known facts (1) that amino acids, when ingested, spare the "basal" protein metabolism and (2) that the N of ingested amino acids is delayed in its elimination even though the metabolism of the non-nitrogenous portion be under way. Furthermore, when the dog was made diabetic "extra sugar" appeared in the urine of the hours under consideration to an extent which indicated a metabolism of glutamic acid which was the equivalent of 0.2 gm. of N per hour.

In the computations we have assumed that the basal (endogenous) protein metabolism corresponded to the equivalent of 0.1 gm., and that of glutamic acid to 0.2 gm. of N per hour.

The deductions from the values of the respiratory gases obtained in the experiments on the normal dog would therefore be:

	O ₂	CO ₂	Calories.
	gm.	gm.	
For 0.1 gm. basal urine N.....	0.849	0.935	2.651
“ 0.2 “ glutamic acid N.....	2.064	2.836	6.610
	2.91	3.77	9.26

However, it makes little difference in the end result whether the metabolism is calculated with the use of the foregoing factors or whether the customary values for protein are employed. Thus in Experiment 12, if the metabolism of 3 hours be calculated as a whole, with the factors given above, 76.26 calories are obtained, whereas if the usual protein values are employed 76.56 calories are the result. The non-protein R.Q.'s, however, are respectively 0.85 and 0.91.

In the diabetic condition the protein and glutamic acid metabolisms were calculated in accordance with the following principles:

Experiment 15 A.

- a. Total urine N 0.542 gm. — 0.02 gm. Liebig's extract N = 0.522 gm. N per hr.
- b. D eliminated per hr. = 2.695 gm.
- c. $0.522 \text{ gm. N} \times 3.65 = 1.905 \text{ gm. usual diabetic D.}$
- d. Difference or extra D per hr. = 0.79 gm.
- e. $7.25 \text{ gm. D to 1 gm. glutamic acid N} - 3.65 \text{ gm. D from protein} = 3.6 \text{ gm. extra D per gm. glutamic acid N metabolized. Therefore, } 0.79 \text{ gm. extra urine D} \div 3.6 = 0.219 \text{ gm. glutamic acid N metabolized per hr.}$
- f. $0.522 \text{ gm.} - 0.219 \text{ gm.} = 0.303 \text{ gm. basal diabetic N per hr.}$

Experiment 16 A. — Values are calculated as in (a) to (f), Experiment 15 A.

- a. $0.491 \text{ gm.} - 0.02 \text{ gm.} = 0.471 \text{ gm.}$
- b. 2.50 gm.
- c. $0.471 \text{ gm.} \times 3.65 = 1.719 \text{ gm.}$
- d. 0.76 gm.
- e. $\frac{0.76 \text{ gm.}}{3.6} = 0.217 \text{ gm. glutamic acid N.}$

f. 0.471 gm. - 0.217 gm. = 0.254 gm. basal diabetic N.

Experiment 18 A.—In this the D:N has fallen to 3.0 and a computation indicates that the glutamic acid N is 0.174 gm., while the quota belonging to the basal N is 0.267 gm.

Table III illustrates once more the well known protecting power of ingested amino acids upon protein metabolism in diabetes.

EXPERIMENTAL PART.

The plan of study applied to Dog 61 in Series I (Table IV) involved its maintenance upon a standard diet administered daily at 5 p.m.; the determination of its basal metabolism, the influence

TABLE III.
Urinary N in Diabetes with or without Glutamic Acid.

Experiment No.		Total N.	Basal N.	Glutamic acid N.	D:N
		gm. per hr.	gm. per hr.	gm. per hr.	
15	Basal.	0.46	0.46		5.05*
15 A	Glutamic acid.	0.52	0.30	0.22	4.97
16	Basal.	0.45	0.45		3.72
16 A	Glutamic acid.	0.47	0.25	0.22	5.09
18	Basal.	0.44	0.44		3.14
18 A	Glutamic acid.	0.44	0.27	0.17	4.47

* Morning urine obtained from the bladder before this period showed a D:N of 3.78.

of the ingestion of 10 gm. of lard (two experiments), the influence of 20 gm. of glutamic acid (one experiment), of 20 gm. of glutamic acid plus 10 gm. of lard, of 300 gm. of meat. Except when meat was given 1 gm. of Liebig's extract of beef was added in 100 cc. of water to give flavor to the other diets. In Series II the dog had its basal metabolism determined on the 4th day of fasting and on the 3rd day of phlorhizin¹ glycosuria (induced by the daily subcutaneous injection of 1 gm. of phlorhizin suspended in 10 cc. of olive oil). Thereafter, to the dog, now rendered completely diabetic, the glutamic acid-fat mixture was given in the same amount as when

¹ Lusk has elsewhere (*Ergebn. Physiol.*, 12, 315 (1912)) asked that this spelling of "phlorhizin" be adhered to and he has quoted Lépine as saying, "En tout cas au point de vue de l'étymologie phloridzin est une barbarisme."

TABLE IV.
Metabolism Experiments upon Dog 61.

Experiment No.	Sci										Direct.	Indirect.	Body weight.
	1949		No. of hrs.	CO ₂			Urine N.	D:N	Non-protein N.g.				
				Basal.	gm.	gm.				gm.	gm.		
1	Feb. 11		Basal.	2	7.35	6.54	0.169	0.82	0.82	21.9	21.4	12.0	
4	" 15		"	3	7.32	6.58	0.133	0.81	0.81	21.9	21.7	11.8	
10	Mar. 2		"	3	7.99	6.42	0.152	0.87	0.88	21.7	20.7	13.1	
14	" 7		"	1	7.58	6.45	0.161	0.86	0.86	21.9	20.8	13.2	
Average.....							0.154			21.8	21.2		
7	Feb. 20		Lard, 10 gm., 3rd, 4th, 5th hrs.	3	7.58	6.64	0.107	0.82	0.83	22.3	22.0	12.2	
11	Mar. 4		" 10 " 3rd, 4th, 5th "	3	7.67	6.85	0.166	0.82	0.82	22.8	23.9	13.0	
Average.....							0.137			22.6	23.0		
8	Feb. 21		Glutamic acid, 20 gm., 3rd hr.	1	8.08	6.46	0.185	0.91	0.88	21.4	20.0	12.2	
5	Feb. 18		Glutamic acid, 20 gm., + lard, 10 gm., 4th, 5th hrs.										
9	Mar. 1		Glutamic acid, 20 gm., + lard, 10 gm., 3rd, 4th hrs.	2	8.84	7.94	0.225	0.84	0.78	25.0	22.1	11.9	
12	" 5		Glutamic acid, 20 gm., + lard, 10 gm., 3rd, 4th, 6th hrs.	2	10.04	7.36	0.262	0.99	1.02	25.0	24.7	13.1	
Average.....				3	9.18	7.56	0.273	0.88	0.85	25.1	23.2	13.0	
Average.....							0.253			25.0	23.3		
14 A	Mar. 7		300 gm. lean meat 4th, 5th hrs.	2	11.66	10.33	0.837	0.82	0.86	33.2	33.3	13.2	
										+15.0%			
										33.2	33.3	13.2	
										+52.3%			

Series II. Dog, diabetic.

[illegible]

the animal was normal and the other diets described were likewise administered to the dog when diabetic.

These results are summarized in Table IV and Chart I.

Analysis of Results

A. In the Normal Dog.

Basal Metabolism.—Notwithstanding the fact that an increase in the quantity of fat and carbohydrate, administered in the basal

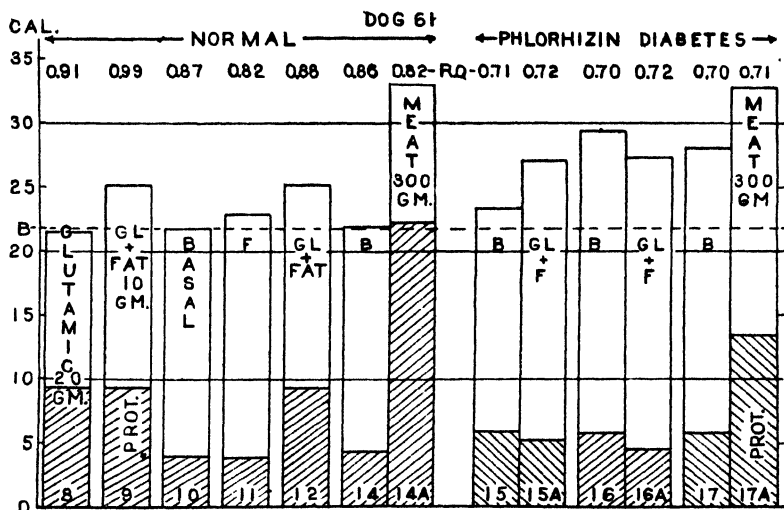


CHART I. Showing the influence of various foodstuffs upon the metabolism of the normal dog and the same dog made diabetic with phlorhizin. GL = glutamic acid; F = fat (lard); B = basal metabolism. Experiments bearing the same numeral, *i.e.*, 14, 14 A, were carried out on the same day. The etched part of the column represents the energy liberated from protein and from glutamic acid.

ration after February 27, caused a gain in body weight, Dog 61 manifested a remarkable constancy in basal metabolism, averaging 21.8 calories per hour, with \pm variations of 0.1 calorie during a period of a month. Mr. Herbert Pollack computed for us the surface area of the dog on March 7 when the body weight was 13.2 kilos and the body length 81.5 cm. The Cowgill-Drabkin (7) formula gave an area of 0.5147 sq. m., indicating the heat produc-

tion of 42.3 calories per hour per sq. m. of surface, which is unusually high (21 per cent above Cowgill and Drabkin's normal average of 34.8 calories).

Lard, 10 Gm.—The administration of this small quantity of lard increased the heat production by 0.8 calorie per hour, or 3.5 per cent.

Glutamic Acid, 20 Gm.—As in former experiments from this laboratory, the administration of glutamic acid failed to change the metabolism appreciably (−0.4 calorie per hour, or −1.8 per cent).

Glutamic Acid, 20 Gm., Plus Lard, 10 Gm.—When glutamic acid was given with lard and Liebig's extract soup the metabolism appreciably increased. The dog produced 25.0 calories with great constancy of reaction in three different experiments, an increase of 3.2 calories per hour or 15 per cent. This may be due to an increase in the number of metabolites available for oxidation.

Meat, 300 Gm.—When lean heart meat was given the gm. of N in the urine rose from a basal level of 0.154 to one of 0.837 per hour, an increase of 0.683, which is the equivalent of an increase in protein metabolism of 18.0 calories. The heat production reached 33.2 calories, an increase of 11.4 calories or 52.3 per cent. Of 100 calories of extra protein metabolized 66 appeared as extra heat, a high specific dynamic action.

B. In the Diabetic Dog.

Basal Metabolism in the Fasting Phlorhizinized Dog.—The average R.Q. in four experiments, made on successive days, the 4th to 7th of phlorhization, was 0.705, and the non-protein R.Q.'s averaged 0.724, which is closely akin to the R.Q. for fat. The maximum heat production, 29.4 calories, occurred on the 4th day and was 35 per cent above the normal basal level. From this point it fell steadily to 24.8 calories on the 7th day, due perhaps to the progressive emaciation of the dog.

Lard, 10 Gm.—The administration of lard caused a heat production of 26.2 calories per hour, accompanied by an R.Q. of 0.699 and a non-protein R.Q. of 0.717 during the 3rd and 4th hours after its administration. The heat production contrasts with a basal metabolism of 24.8 calories on the same day as the experiment, and one of 26.7 calories the day previously. Possibly the added

fat contributed fuel to the emaciated organism, enabling it to produce heat, as it had done the day before. (See next paragraph.)

Glutamic Acid, 20 Gm.; Lard, 10 Gm.—When this was given with Liebig's extract of beef in two of the three experiments the R.Q. was 0.719 and the non-protein R.Q. 0.713. The results of the determination of the heat production were as follows:

Experiment No.	Basal metabolism.	After food mixture.
	<i>cal.</i>	<i>cal.</i>
11	23.4	27.0
12	29.4	27.3
14	26.7	25.1
Average.....	26.5	26.5

Although the results are irregular, the average figures indicate that there is no specific dynamic effect in diabetes when glutamic acid and fat are given together.

Meat, 300 Gm.—After giving meat to the dog the heat production was determined during those hours when it had risen to its maximum. The R.Q. in two instances was 0.709 and the non-protein R.Q. 0.77. (*Note.* In a similar experiment on Dog 65, given in Table V, the R.Q. was 0.700 and the non-protein R.Q. was 0.728, or essentially that of fat.) The increases in metabolism following protein ingestion may thus be analyzed:

Experiment No.	Basal metabolism.	After meat.	Increase.	Increase in urine N.	Increase in calories + increase in calories of protein metabolism.
	<i>cal.</i>	<i>cal.</i>	<i>cal.</i>	<i>gm.</i>	
17, 17 A	28.4	32.7	4.3	0.6	27
19, 20	24.8	29.9	5.1	0.6	32

The increase in gm. of urinary N has been multiplied by the factor 26.51 calories normally potential in the protein metabolism represented by 1 gm. of urinary N. The 15.9 calories so obtained, divided into the increase in total metabolism, gave a specific dynamic action of 30 per cent, in contrast with 66 per cent obtained when the dog was normal.

More striking than this, however, is the fact that, whereas in the

TABLE V.
Food in Phlorhizin Diabetes.

Dog 65. April, 1929. Body weight, 12.1 to 11.1 kilos.

Experiment No.	Nature of experiment.	CO ₂ per hr.	O ₂ per hr.	Urine N per hr.	D:N	R.Q.	Non-protein R.Q.	Calories per hr.		Extra D per hr. gm.
		gm.	gm.	gm.				Indirect.	Direct.	
1	Basal.....	8.19	8.36	0.337	3.41	0.713	0.725	26.77	24.82	
1 A	Fat, 10 gm., + glutamic acid, 20 gm.....	8.41	8.34	0.417	4.05	0.734	0.741	26.57	29.40	0.38
2	Basal.....	8.37	8.84	0.424	3.26	0.689	0.697	28.08	24.08	
2 A	Fat + glutamic acid.....	8.01	7.85	0.477	4.03	0.719	0.755	24.93	26.77	0.41
3	Basal.....	7.26	7.67	0.349	3.61	0.688	0.697	24.41	24.34	
3 A	Meat, 300 gm....	11.91	12.37	1.008	3.20	0.700	0.728	38.50	36.50	
Average.....								28.21	27.65	

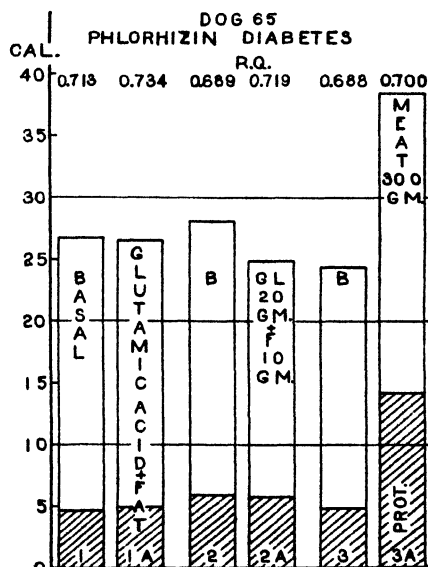


CHART II. Showing the influence of various foodstuffs upon the metabolism of a phlorhizinized dog. The abbreviations are the same as on Chart I.

normal dog the heat production rose to 33.2 calories (0.84 gm. of N in urine per hour), in the same dog on the 5th day of fasting and diabetes the heat production reached 32.7 calories (1.05 gm. of N in urine per hour). It would seem, therefore, that the specific dynamic action of protein is approximately the same in the two instances.

The evidence obtained from Dog 65 during phlorhizin diabetes may be found in Table V and Chart II. It is so absolutely concordant with that obtained from Dog 61 that it requires no further analysis.

DISCUSSION.

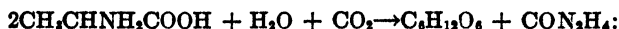
When contrasted with the behavior of glycine and alanine, it is evident that glutamic acid exerts no specific dynamic action either in the normal or in the diabetic dog. Also it appears that, when meat is given to the normal dog and to the same animal rendered diabetic with phlorhizin, the heat production in both instances reaches the same level. Since the increase in metabolism is the same after giving glycine to the normal and to the diabetic dog, it would appear that the fact that glucose, arising from either protein or glycine, remains unoxidized, has no bearing on the specific dynamic action of these materials.

Lusk (8) has calculated² that if 2 mols of alanine are deaminized, with the production of ammonia and pyruvic acid, and if pyruvic acid is converted into glycogen, intermediate reactions which liberate or absorb 176 calories are involved. Since the physiological heat value of 2 mols of alanine in metabolism is 593 (*i.e.*, alanine 776—NH₃ 183), it would appear that energy amounting to 30 per cent of that contained in alanine would be used to accomplish the transformation process. This analysis suggested itself from reading the papers of Aubel (9) and of Meyerhof, Lohmann, and Meier (10).

Aubel (11) has calculated that if 1 molecule of alanine passes through the stages of pyruvic and lactic acids into glucose and urea, 113 calories would be required to effect the transformation. If the physiological heat value of alanine in metabolism is 296 calories per mol, then its specific dynamic action would represent 38 per cent of its energy content. A revision of this method of calculation

² The calculations unfortunately are erroneously printed, but the end result is correct.

may be necessary in the light of the work of Adams (12), who has applied the principles of thermodynamics to the solution of the problem. Adams writes as follows regarding Lusk's formula representing the transformation of alanine into glucose,



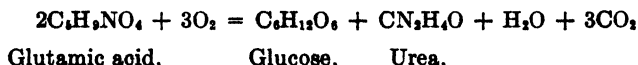
"It is a consequence of the second law [of thermodynamics] that if this change in state does not take place spontaneously, work will be required to make it take place. With the aid of the third law it is possible to show that approximately 60,000 small calories of work will be required to make the change in state take place. . . . In order to do this work fuel must be burned in the organism. . . . the fuel burned may be much larger than the minimum amount required to furnish the necessary work. . . . From this point of view the specific dynamic action of alanine is analogous (perhaps only in part) to the recovery process in muscle."

Adams states also that Lusk's equation for the conversion of glycine into glucose,



by the aid of the third law requires 196,000 small calories. Therefore, per mol of glucose formed, the free energy increase is greater for the metabolism of glycine than for alanine. The calculations were made on the basis of a temperature of 25°, but otherwise the conditions were similar to those existing in phlorhizinized dogs.

Further analysis by Adams, quoted with his permission by Lusk at the recent International Physiological Congress at Boston, showed that ΔF (free energy increment) is negative for the conversion of glutamic acid into glucose according to Lusk's equation,



Hence it is thermodynamically possible for the reaction to take place without the expenditure of work upon the reacting system.

Lusk (5) has calculated the physiological heat value of glycine and alanine and has determined that the heat of the specific dynamic action of glycine is 100 per cent and of alanine 50 per cent of the physiologically available energy within them (1 gm. of glycine = 2.10 calories; 1 gm. of alanine = 3.55 calories). Adams' calculations show that the laws of thermodynamics demand a

minimum of 0.871 calorie to drive the reaction of 1 gm. of glycine into glucose and urea and a minimum of 0.337 calorie to drive 1 gm. of alanine into sugar and urea. These are minima and, just as external muscle work may require a 3-fold energy production for its accomplishment, so this intermediary chemical work may be associated with the production of free heat.

The figures given by Adams indicate a minimal energy quantum of 10 per cent of that physiologically available in 1 gm. of alanine in order to drive it into glucose and urea, whereas the specific dynamic action of alanine is 50 per cent of the available calories in the material. In the case of glycine the minimum quantum of energy necessary to drive it into glucose and urea is 41 per cent of the available calories in the material, whereas the specific dynamic action of glycine, as measured by the liberation of extra heat in metabolism, is 100 per cent.

This work of Adams explains in part, if not wholly, the specific dynamic effect of protein in the simplest way. It is evident why the heat of this chemical energy necessary for the conversion of fragments into glucose cannot be used for mechanical work and why the energy for mechanical work derived from the oxidation of sugar or fat is always to be sharply differentiated from that portion of energy derived from the specific dynamic action of protein.

In this regard it is interesting to note that Bornstein (13) has observed that the perfusion of a liver with blood containing glycine raises the oxygen consumption of this organ by 30 per cent. Glycine disappears from the circulating blood, and urea and ammonia increase therein. Bornstein (14) also states that, after perfusing the isolated muscles of the extremities with blood containing glycine, there is no increase in oxygen consumption in some instances, though an average increase of 5 per cent is observed. This work recalls the observations of Aub and Du Bois (15) upon legless men and achondroplastic dwarfs, which showed that the specific dynamic action of meat was greater per unit of surface area than in normal controls, was dependent on the amount of protein metabolized, and was entirely independent of the mass of muscle tissue.

After giving 10 gm. of fat to the normal dog the metabolism rose 3.5 per cent, but when it was given to phlorhizinized dogs in association with glutamic acid there was no indication that it effected

a rise in metabolism. The known influence of diabetes is to increase the fat supply to the tissues and in the blood, and further contribution to the already existing plethora of fat particles should have no effect on metabolism.

An excellent article on the subject of the specific dynamic action of carbohydrate, written by Hanns Baur (16), has recently appeared, which concludes that the specific dynamic action of carbohydrates is not to be entirely explained by the presence of a carbohydrate plethora. The material therein published appears, however, greatly to strengthen that view, though this is not the place for its detailed discussion.

SUMMARY.

1. In the dog the specific dynamic action of glycine, as measured by its calorogenic action, is dependent on the amount administered and is independent of the size of the animal which has received the glycine.

2. Glutamic acid given to the normal or diabetic dog exerts no specific dynamic action.

3. Fat given in small quantity to the normal dog shows a slight specific dynamic effect and may show none in the diabetic dog.

4. The specific dynamic action of meat appears to be the same in the normal as in the diabetic dog. Just as in the case of a fasting dog, which in running maintains the reaction glycogen-lactic acid-glycogen at the expense of fat which is oxidized, so the fuel which is necessary to drive certain of the cleavage products of protein into glucose in diabetes may be fat. The fuel needed for this purpose wholly or in part constitutes the extra heat of the specific dynamic action of protein. This analysis accords with the ideas of Aubel, of Meyerhof, and of Adams.

We wish to express our obligation to Dr. Herbert Pollack, who as a fourth year medical student rendered valuable aid in the experiments and in the formulation of the theories advanced in this paper.

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THE ARGINASE METHOD FOR THE DETERMINATION OF ARGININE AND ITS USE IN THE ANALYSIS OF PROTEINS.*

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The use of the enzyme arginase for the quantitative determination of arginine was first proposed in 1917 by Jansen (1). His procedure, as applied to the analysis of proteins, involved the simultaneous addition, to a slightly alkaline hydrolysate, of arginase and urease. By the first of these enzymes any arginine present was split into ornithine and urea, by the second the urea was converted into ammonium carbonate. After a suitable interval (24 hours) the ammonia thus produced was determined by the usual process of aeration and titration. A control conducted with urease alone furnished the necessary correction for preexisting ammonia and urea.

Jansen described his method rather briefly, and the experience of this laboratory, dating from 1922 (2), has indicated that its successful employment requires attention to many details which its author left unmentioned. These details mastered, the method has been found to be not only, as was to be expected, strictly specific, but also highly accurate. Some of the earlier results which we obtained with it were communicated to the American Society of Biological Chemists in 1924 (3), others to the Royal Society of Canada in 1925 (4). We have had it in constant use ever since, and feel that we can now confidently recommend it as an analytical procedure at once trustworthy and convenient.

The central principle of the method—the use of arginase as a quantitative reagent—has in the meantime been employed, in

* Most of the experimental data of this paper are taken from a thesis presented by James A. Dauphinee in partial fulfilment of the requirements for the degree of Doctor of Philosophy at the University of Toronto.

different ways, by others. Iwanoff (5) in 1925 showed that arginine could be split by a living culture of *Aspergillus niger*, yielding in 5 days 92 to 94 per cent of the theoretical amount of urea; and he attempted, not altogether without success, to apply this observation in the analysis of protein. His technique, requiring, as it does, from 5 to 11 days of incubation with the mold, is obviously unsuited for general analytical work. Much more attractive is the procedure described in 1927 by Bonot and Cahn (6). These authors employed a dried arginase powder obtained from dog liver press juice by acetone precipitation, allowed it to act upon hydrolyzed protein or tissue for 72 hours at pH 9.9, and determined the urea, as Iwanoff also had done, by the xanthidrol method of Fosse. They claim for this method, doubtless with justice, an accuracy within 2 per cent; but it may be objected that 3 days is still an inconveniently long period of incubation, that the determination of urea by xanthidrol requires a preliminary concentration *in vacuo*, and that the use of a gravimetric process has in any case its own evident disadvantages. We have had no difficulty in securing arginase preparations that will hydrolyze adequate quantities of arginine in 12 to 24 hours; while by taking appropriate controls we meet readily enough certain objections offered by Bonot and Cahn to the use of urease, and retain all the conveniences of a titrimetric procedure.

It is the purpose of the present paper to discuss in detail the precautions necessary for the successful employment of Jansen's principle; to describe, with special reference to the determination of arginine in proteins, the precise technique which we have been finally led to adopt; and to show that this does indeed yield results of a satisfactory degree of precision.

I. Preparation and Testing of Arginase Solutions.

As the most convenient source of arginase we have used mammalian liver—generally that of the ox or calf, but sometimes that of the pig. From such material the arginase may be extracted by water, dilute acetic acid, or glycerol. The extracts contain, of course, much extraneous material—protein, miscellaneous enzymes other than arginase, amino acids (including doubtless arginine itself), urea, ammonia, etc. From much of this material the enzyme can be freed by alcohol, alcohol-ether, or acetone precipi-

tation. Unfortunately such treatment has resulted almost invariably, in our experience, in a considerable loss of activity. We abandoned therefore, after many fruitless experiments, every attempt to purify the enzyme, and reverted to the use of extracts of which the comparative crudity was compensated by a high degree of activity. As the medium of extraction we have used both water and glycerol, but most frequently the latter. Glycerol extracts have the advantage over aqueous ones of resisting the growth of bacteria or molds, and of retaining almost their full activity for a long period of time.

1. *Method of Measuring Arginase Activity.*—In studying the comparative efficiency of various methods of preparing the extract, as well as in ascertaining whether any given extract was active enough for the purpose in hand, it was useful to have a rapid yet accurate method of measuring arginase content. For this purpose we used a procedure identical in principle with that which we have already employed in another connection (7).

First of all a graph was prepared connecting known volumes of an arginase solution, and therefore known relative quantities of the enzyme, with the amounts of arginine which they decomposed in a given time under certain set conditions. This was done in the following way.

A neutralized solution of arginine was prepared of such strength that 5 cc. contained 31.1 mg. of the base or 10 mg. of nitrogen. 2 cc. of an active liver extract were diluted to 20 cc., and placed in a water thermostat at 37°. Into each of eight urea tubes were introduced 2 cc. of 0.5 M phosphate buffer solution of pH 8.4. This was followed in the case of four tubes by 5 cc. of the arginine solution, in that of the other four (which served as controls) by 5 cc. of water. The tubes were then placed in the thermostat. When all had reached the temperature of the bath, one arginine tube and one control tube were treated with 0.5 cc. of the diluted liver extract, the time of the addition being carefully noted. A second pair of tubes received 1.0 cc., a third 2.5, and a fourth 5.0. Exactly 30 minutes after the addition of the enzyme in each case the tubes were removed from the bath and their contents boiled. They were then cooled, neutralized to pH 6.8, and submitted to the Van Slyke-Cullen process for the determination of urea (8). The results, corrected by the blanks, were plotted in Chart 1.

From this graph we now selected, as a convenient arbitrary unit of arginase, that amount which, under all the conditions of the experiment, produced urea equivalent to 0.5 mg. of nitrogen, or which, to express it otherwise, decomposed 10 per cent of the total arginine present.¹ A scale of units on this basis having been laid down along the axis of abscissæ, we had a curve showing at once the relation between action (under the chosen conditions) and arginase content (in the adopted units). With this at hand

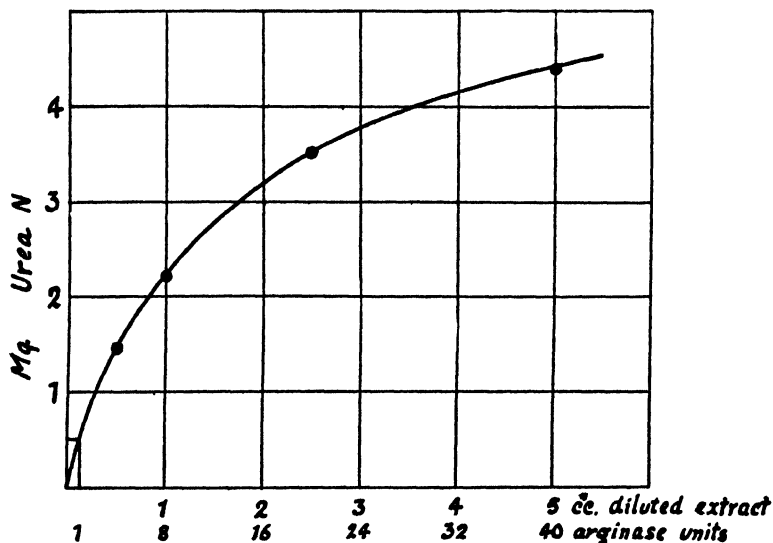


CHART 1. Curve used for the estimation of unit amounts of arginase in liver extracts. (1 unit of arginase = amount required to liberate 0.5 mg. of urea N from 10 mg. of arginine N in $\frac{1}{2}$ hour at 37° and pH 8.4.)

it was easy to find a quantitative expression for the arginase content of any particular extract. All that was necessary was to dilute the extract to an appropriate extent (say ten times), and to determine, in the precise manner just described, the action of 1 cc. (or any other convenient quantity). The amount of urea nitrogen formed in the prescribed half hour gave then at once,

¹ This is a unit different from, and much larger than, the one used in our work on arginase distribution (7).

on reference to the graph, the number of arginase units in the volume used.²

The diluted extract used in constructing Chart 1 contained, it will be seen, 8 units, the original extract therefore 80 units, per cc.

2. *Clarification of Liver Extracts by Heating.*—Our technique for the determination of arginine calls for two colorimetric adjustments of reaction. It is important therefore that the solutions used, including the arginase extracts, be perfectly clear and not too deeply colored. Liver extracts are always very turbid and cannot be cleared by centrifugation. Plain aqueous extracts filter very slowly, while glycerol extracts, in their original state, are so viscous as to be practically unfilterable. Fortunately, as we have found, even glycerol extracts become manageable if part of their protein is first coagulated by heat; and, if the heating is properly controlled, it need cause little, if any, destruction of arginase. The conditions which secure the best result are indicated in the following experiment.

Five 25 cc. portions of a crude glycerol extract of liver were diluted each with 25 cc. of water. One was left at room temperature (20°), the others were placed in water baths maintained at 37°, 50°, 60°, and 70° respectively. No coagulation was noticed at 37° or 50°. At 60° and 70° coagulation was perceptible as soon as the mixture reached the temperature of the bath, and seemed to be complete within 5 minutes. After this interval the contents of each tube were thrown on a folded paper filter. The first three (held at 20°, 37°, and 50°) filtered with extreme slowness, yielding a deep red filtrate containing (as determined by the method just described) 35 units of arginase per cc. The one heated to 60° passed the filter much more rapidly. The filtrate was perfectly clear, still red in color, and contained 33 units. The 70° sample gave rapidly a straw-yellow filtrate possessing an activity of only 4.5. Heating for a few minutes to 60° causes therefore little, if any, destruction of the enzyme, yet removes enough protein to cause a great increase in the rate of filtration. At 70° the coagulation of protein is much more complete but within 5 minutes the enzyme is nearly destroyed.

² In all essentials this method of determining arginase is the same as that already described by Edlbacher and Röthler (9); but the unit chosen by these writers was smaller than the one employed here.

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Although the coagulum produced at 60° seems rather bulky, it includes only a small fraction of the total protein. Thus, in another experiment an aqueous extract, of which 1 cc. contained 6.43 mg. of nitrogen, was held for 3 minutes at 58°. The filtrate still contained 5.45 mg. of nitrogen per cc. The arginase titer had fallen in the meantime only from 112 to 104. The treatment had removed 15 per cent of the total nitrogen and 7 per cent of the enzyme.

3. Optimum Period of Extraction.—In the preparation of endocellular enzymes it is common practice to extract a tissue for a period of at least 24 hours. The following experiment will show that in the case of arginase this is not only unnecessary, but actually disadvantageous.

A pound of liver, purchased in the market, was finely ground, and six lots of 25 gm. each were weighed out into large test-tubes. To each were added 50 cc. of water, and all were vigorously shaken. One tube was then immediately heated to 60° in a water bath, maintained at this temperature for 2 minutes, cooled, and filtered. To one of the remaining tubes there was added a small quantity of trypsin; to all 5 cc. of toluene. These tubes were then held at 37° for varying lengths of time, each, after its measured period of extraction, being heated to 60° for 2 minutes and filtered like the first. As each filtrate was obtained, its arginase content was determined in the way already described. The result was somewhat surprising. The first filtrate, obtained after extracting for a few minutes only, contained 88 arginase units per cc., while longer periods of extraction gave not more arginase, but less. Thus after 5 hours the yield was 86, after 20 hours only 70, after 48 hours 62, and after 336 hours 28. The mixture to which trypsin had been added gave, after 20 hours, a filtrate containing only 8 units. This would suggest that the gradual disappearance of arginase during prolonged extraction is due to the action of proteolytic enzymes in the liver itself. However this may be, the experiment shows quite definitely that optimal yields of arginase are to be obtained by a very brief period of extraction.

4. Standard Method of Preparing Crude Arginase Extracts.—The intimations given by the experiments already described led to the adoption of the following as our standard procedure for the preparation of an arginase extract.

A weighed quantity of finely ground fresh liver is mixed in a flask with a volume of 75 per cent glycerol equal in cc. to the weight of the tissue in gm. The mixture is thoroughly shaken for 10 minutes, and placed in a water bath at 62–65°. The flask is held in the bath, with frequent shaking, until its contents have reached a uniform temperature of 58°, kept there for 5 minutes longer, and cooled under the tap. Its contents are then thrown on a large folded filter of Chardin paper. If the heating has been properly conducted, filtration will be fairly rapid, so that if one has taken, for example, 1500 gm. of liver, one should obtain within 12 hours 1000 cc. of a perfectly clear, red filtrate. This filtrate is brought to pH 7 by the addition of sodium hydroxide. Before using it, it is well to determine its activity, which should lie between 80 and 100 units per cc. When not in use, the extract is kept in the ice box.

5. *Preparation of Dried Enzyme Powder.*—It is sometimes convenient to keep and to use the enzyme in dry form. When this is desired the extraction is made with water instead of glycerol, and the filtrate is spread in thin films upon glass plates, of which the edges are thickly smeared with vaseline. Evaporation is hastened by a current of air from a fan, and, when the films are dry, they are scraped off, further desiccated *in vacuo* over H_2SO_4 , and finally reduced to powder. The powder is practically completely soluble in water, and retains for a long period the full activity of the original extract.

6. *Amount of Enzyme Requisite for Determination.*—In working out the application of the arginase-urease method to the analysis of proteins we decided to be satisfied with no procedure which was not capable of determining the arginine equivalent of at least 5 mg. of urea nitrogen (corresponding to 25 cc. of $\text{N}/70 \text{ NH}_3$), or which for such an accomplishment required more than 24 hours of arginase action. The question then arose how much arginase it would be necessary to use to meet these conditions. The answer was found in an experiment performed at the same time, and on the same general lines, as the one used (see p. 629) in the preparation of Chart 1. The only differences were that each tube, in a series of five, received here the same quantity (1 cc. = 8 arginase units) of the diluted liver extract, and that the action was allowed to proceed for varying intervals from 0.5 to 22 hours. The results are shown in Table I.

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From these results it is evident that under the conditions imposed in the experiment (temperature 37°, pH about 8.4, and m/8 concentration of phosphate) 8 units of arginase suffice to decompose completely in about 15 hours as much arginine as will yield 5 mg. of urea nitrogen. This performance more than satisfies the prescribed requirements. In order, however, to deal safely with even larger quantities of arginine, in order also to provide against the possible presence of unknown inhibitants, we make it a practice to use in actual analyses as much as 1 cc. of undiluted extract. This will furnish as a rule 80 units or more of arginase, which would appear to be, under any probable conditions, a sufficiently large excess.

TABLE I.

Action of 8 Units of Arginase upon 31.1 Mg. of Arginine (Containing 10 Mg. of Nitrogen).

Time of arginase action.	Urea N found.	Arginine decomposed.	
hrs.	mg.	mg. N	per cent of total
0.5	2 26	4 52	45.2
1.0	3.01	6.02	60.2
4.0	4 51	9.02	90.2
10.0	4 97	9.94	99.4
22 0	5.00	10.00	100.0

II. Determination of Arginine in Pure Solution. Limit of Accuracy of the Arginase-Urease Method.

Obviously, before arginase may be used for the routine determination of arginine, one must be assured that it is capable of hydrolyzing that substance in a quantitative manner. Jansen appears to have taken the point for granted. Among those who have reported actual experiments with pure arginine Iwanoff (5) obtained only 92.5 to 94.1 per cent decomposition, Edlbacher and Bonem (10) not more than 97 per cent, Bonot and Cahn (6) on the other hand 98 to 100 per cent. We ourselves, in a first series of tests, secured results indicating as the maximum attainable degree of hydrolysis 97.7 to 98.7 (average 98.1) per cent. The absolute validity of these results seemed however upon two grounds to be open to doubt. In the first place we had not

measured the rotatory power of our arginine, which might therefore have contained an appreciable admixture of the non-reacting levorotatory form. In the second, the failure to obtain a quantitative result was perhaps to be attributed, either in whole or in part, to incomplete action, not of the arginase itself, but of the urease employed for the determination of urea. We resolved therefore to conduct a second series of experiments with an arginine of known optical purity; and, as a preliminary, to ascertain with what degree of precision we were actually estimating urea.

1. *Accuracy of the Urease Method for Urea.*—Van Slyke and Cullen (8), testing the urease method upon pure solutions of urea, obtained results up to 99.8 to 100.7 per cent of the theoretical; but their duplicates, as Fiske (11) points out, sometimes differed by as much as 1 per cent. Later experiments by Van Slyke (12) show a recovery, by the aeration technique, of only 98.2 to 99.1 per cent. Murray (13), though she used a special apparatus for aeration, got at the most 98.4 per cent. The best results on record are those of Fiske (11), who consistently obtained 99.8 to 99.9 per cent. Unfortunately Fiske's estimates of the amounts of urea dealt with were based not upon direct weighing, but upon nitrogen determinations according to Kjeldahl. Since this procedure introduces at once an element of uncertainty, we felt it necessary, for our own purposes, to submit the urease method to an independent examination.

With this purpose in view two samples of c.p. urea of a well known brand were recrystallized three or four times from water. In order to avoid the partial decomposition which occurs when urea solutions are boiled (14) the temperature during recrystallization was never allowed to exceed 40°. The product of this treatment might, it was thought, be assumed to be perfectly pure urea; but as a control upon its behavior we prepared a specimen of urea oxalate, and submitted this also to three recrystallizations. Before use both the urea and the oxalate were carefully dried to constant weight, either by heating in an oven at 100–105°, or by prolonged vacuum desiccation.

The solutions used in testing the urease method were prepared by dissolving about 0.7 gm. of urea (or a corresponding amount of the oxalate) in enough 0.2 M phosphate solution of pH 6.8 to

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make a total volume of 250 cc. In each test several 5 cc. portions of such a solution were treated according to the technique of Van Slyke and Cullen (8) modified in agreement with the recommendations of Fiske (11). The urease employed was a glycerol extract of jack bean, of which 1 cc. (found by actual test to be an ample excess) was allowed to act at room temperature for 1 hour (no doubt an unnecessarily long period). Aeration, which was controlled by a gas meter, was conducted at a speed rising gradually to 100 liters per hour, and was maintained for at least 2 hours.³ Caprylic acid was used as antifoam. The receivers (large test-tubes of Pyrex glass) were charged with 10 cc. of $N/14$ HCl, prepared from constant boiling point HCl by the method of Hulett and Bonner (15), as improved by Foulk and Hollingsworth (16). The back titration was made from extra long 25 cc. burettes with approximately $N/70$ CO_2 -free NaOH. The indicator used was a mixture of methyl red and methylene green, which gives an exceedingly sharp end-point. During the titration the liquid in the tube was stirred by a current of CO_2 -free air, and was so diluted with neutral CO_2 -free distilled water that the volume in which the end-point was reached was always the same. The normality of the alkali was checked at frequent intervals against the standard acid, the same conditions being maintained as in the titration of an unknown. All volumetric glassware was carefully calibrated, and appropriate corrections were made for the prevailing temperature. With these precautions, most of which have been suggested already by Allen and Davisson (17), we found it possible to measure 6.5 mg. of urea nitrogen with a divergence between duplicates of not more than 0.2 per cent. Suitable controls, containing phosphate and urease but no urea, indicated for each experiment the correction required for traces of ammonia in the reagents.

The results of six experiments, involving twenty-five determinations, are shown in Table II. If one divergent figure (in Experiment 4) be rejected, there remain twenty-four consistent results showing a yield of ammonia between 99.2 and 99.5 per cent of the theoretical. The average percentage recovery is 99.4.

³ Separate check experiments showed that a current of smaller volume or velocity could not be depended upon to expel the last residual traces of ammonia.

The data of Table II, it may be remarked, are not necessarily inconsistent with the higher yields reported by Fiske, for these, as

TABLE II.
Control Experiments with Pure Urea.

Experiment No.	Specimen used.	Calculated amount of urea N in 6 cc. solution.	N/70 acid neutralized (corrected for blank [*]).	Urea N found.	Recovery.
		<i>mg.</i>	<i>cc.</i>	<i>mg.</i>	<i>per cent</i>
1	Urea I.	6.534	32.49	6.498	99.4
			32.52	6.504	99.5
			32.49	6.498	99.4
			32.51	6.502	99.5
			32.44	6.488	99.3
2	Urea I.	6.620	32.89	6.578	99.4
			32.91	6.582	99.4
			32.93	6.586	99.5
			32.88	6.576	99.3
3	Urea II.	6.486	32.22	6.444	99.4
			32.19	6.438	99.3
			32.20	6.440	99.3
			32.21	6.442	99.3
4	Urea II.	6.477	32.13	6.426	99.2
			32.13	6.426	99.2
			32.14	6.428	99.2
			32.02	6.404	98.9
5	Urea II.	6.496	32.33	6.466	99.5
			32.28	6.456	99.4
			32.31	6.462	99.5
			32.27	6.454	99.4
6	Urea oxalate.	6.334	31.46	6.292	99.3
			31.50	6.300	99.5
			31.45	6.290	99.3
			31.45	6.290	99.3

* In Experiment 1, where the urease had been treated with permutit, the blank was 0.05 cc.; in Experiment 2 it was 0.12 cc.; in Experiments 3 to 6, 0.20 cc.

has been stated, were calculated upon the somewhat uncertain basis of a Kjeldahl determination. Whether our failure to secure

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TABLE III.
Control Experiments with Pure Arginine.

Solution No.	Arginine-HCl in 50 cc.	Determination No.	Volume of solution.	Calculated urea N recoverable.	Time of action.	N/70 acid neutralized (corrected for blank).	Urea N found.	Recovery.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
	gm.		cc.	mg.	hrs.	cc.	mg.	per cent
I	0.7727	1	1.019	2.095	24	10.30	2.060	98.3
		2	1.019	2.095	24	10.36	2.072	99.0
		3	1.019	2.095	24	10.34	2.068	98.7
		4	1.019	2.095	24	10.42	2.084	99.5*
		5	1.019	2.095	24	10.34	2.068	98.7
		6	1.019	2.095	24	10.41	2.082	99.4*
		7	1.019	2.095	48	10.29	2.058	98.2
		8	2.022	4.157	24	20.44	4.088	98.4
		9	2.022	4.157	24	20.51	4.102	98.7
		10	3.013	6.194	24	30.37	6.074	98.1
		11	3.013	6.194	24	30.51	6.102	98.5
		12	3.013	6.194	24	30.54	6.108	98.6
II	0.7559	13	1.007	2.025	24	9.94	1.988	98.2
		14	1.007	2.025	24	9.96	1.992	98.4
		15	1.007	2.025	24	10.01	2.002	98.8
		16	1.007	2.025	48	9.95	1.990	98.3
		17	2.022	4.066	24	19.93	3.986	98.1
		18	2.022	4.066	24	20.00	4.000	98.4
		19	2.022	4.066	48	20.03	4.006	98.5
		20	2.022	4.066	48	19.95	3.990	98.2
		21	3.013	6.059	24	29.57	5.914	97.6*
		22	3.013	6.059	24	29.67	5.934	97.9*
		23	3.013	6.059	48	29.97	5.994	98.9
		24	3.013	6.059	48	29.93	5.986	98.8
		25	3.013	6.059	48	29.75	5.950	98.2
III	0.4223	26	4.973	5.588	48	27.24	5.448	97.5*
		27	4.973	5.588	48	27.50	5.500	98.5
		28	4.973	5.588	48	27.59	5.518	98.8
		29	4.973	5.588	48	27.53	5.506	98.6
		30	4.973	5.588	48	27.54	5.508	98.6
Average.....								98.5

* Not included in the calculated average.

a really quantitative recovery means that the reaction does actually fall just short of complete decomposition, or whether it is

merely a measure of the accumulated errors of our technique, we are not now prepared to discuss. It is enough for our purpose that the precise limitations of the method, as we ourselves applied it, have been defined. It is worthy nevertheless of note that both Kay (18) and Mack and Villars (19) have found that the action of urease is, under certain conditions, reversible. The existence here of a real equilibrium would therefore not be surprising.

2. Technique and Accuracy of Arginine Determination As Applied to Pure Solutions.—The experiments upon which we based our final estimate of the accuracy of the arginase method were conducted with a substrate of exceptional optical purity—with that Arginine Hydrochloride I, namely, which has been described by one of us elsewhere (20) as having a specific rotatory power of $+21.95^\circ$. A description of these experiments will incidentally indicate a procedure suitable for the determination of arginine in pure solution.

After the arginine salt had been thoroughly dried at 110° the quantities shown in Column 2 of Table III were dissolved to make in each case a total volume of 50 cc. The solvent used for Solutions I and II was pure water, but for Solution III it was a 0.2 M solution of Na_2HPO_4 . In the experiments with the first two solutions portions of 1, 2, or 3 cc. were measured with calibrated Ostwald pipettes into urea tubes, to which were further added 2 cc. of 0.5 M Na_2HPO_4 and 2, 1, or 0 cc. respectively of water. Each tube received in this way a total of 5 cc. of liquid having a phosphate concentration of 0.2 M. From Solution III, which already contained phosphate, 5 cc. portions were measured directly. (The precise volume of arginine solution delivered in each case by the particular pipette used is shown in Column 4, Table III.) To the contents of each tube there were added 2 cc. of arginase solution (in this case a glycerol extract of dried liver tissue), a drop of phenolphthalein indicator, and enough 0.5 N NaOH (2 or 3 drops) to give a distinct but not too deep pink color. This meant that the mixture was brought to a pH of about 8.5.⁴

⁴ As stated by Hunter and Morrell (21) and by Edlbacher and Bonem (10), the optimum pH for the initial action of arginase is 9.8. But we have found, in experiments not yet published, that at this reaction the enzyme undergoes fairly rapid destruction, so that after a sufficiently long interval (such as that allowed in an arginine determination) the total amount of

It was then treated with a little toluene and left, lightly covered, at room temperature for 24 or, in some cases, 48 hours.⁵ At the end of this period a drop or two of phenol red indicator was added, followed by as many drops (5 or 6) of 5 per cent HCl as were necessary to change the color to a bright yellow. The mixture was then boiled in order to coagulate the protein, cooled, and titrated with 10 per cent NaOH to a commencing pink (pH 6.8). 1 cc. of an active urease extract was added, and the urea was determined by the technique described in the preceding section.

With each group of analyses four or five controls were run, in each of which 5 cc. of 0.2 M phosphate solution (without any arginine) were incubated with arginase and subsequently with urease under the same conditions as in the main experiment. The average results of these controls varied, at different times and with different samples of arginase, from 0.78 to 1.07 cc. of N/70 ammonia.

The data of several series of determinations made upon quantities of arginine yielding from 2 to 6 mg. of urea nitrogen are brought together in Table III. On examining Table III it will be seen that, when not more than 4 mg. are involved, the result is no higher after 48 hours of arginase action than after 24; so that even after the shorter interval the reaction had indubitably run to completion. With 6 mg. of nitrogen this is not always the case. It may perhaps be assumed to have been so in Determinations 10 and 11, which, incidentally, were made on an unusually warm day; but a comparison of Determinations 21 and 22 with Determinations 23 to 25 shows that in this group a period of 24 hours was too short. For this reason Determinations 21 and 22 are neglected in drawing the average. Determinations 4, 6, and 26 may be rejected as giving evidently erroneous results. The twenty-five

arginine decomposed is likely to be almost as great at 8.5 as at 9.8. For this reason we have preferred to work at the lower alkalinity, which it is easier in practice to define by colorimetric means.

⁵ For the determination of an unknown it is generally more convenient to work at 37°, and to use but 1 cc. of the heated extract of fresh liver already described. Under these conditions the time of action may be reduced to 12 to 24 hours. It should be said that the extract of dried liver used in the present series of experiments was much less active than the fresh extracts which we now recommend.

determinations remaining all yield results between 98.1 and 99.0 per cent of the theoretical. Those made with Solution I give an average of 98.5 per cent; those with Solution II, 98.4; those with Solution III, 98.6. The average of all is 98.5 per cent.

Remembering that the urease method indicates only 99.4 per cent of the urea actually present, one may calculate that the apparent decomposition of 98.5 per cent of the arginine implies an actual decomposition of 99.1 per cent. As with the urease reaction itself, one is left uncertain whether the indicated deficit (here about 1 per cent) implies the existence of a real equilibrium, or whether it is to be attributed entirely to unavoidable technical errors. There is indeed, in this instance, even a third possibility, for our *D*-arginine, with all its exceptional purity, may still have contained an admixture of its stereoisomer. If this last possibility be neglected, the data, whatever their real significance, may be held to justify the application, to values found by the successive use of both enzymes, of an empirical correction of + 1.5 per cent. Such a correction would bring all the accepted results of Table III to within 0.5 per cent of the truth.

3. Effect of Using Arginase and Urease Simultaneously.—The procedure followed in the experiment just described differed from Jansen's chiefly in the fact that the two enzymes were permitted to act in succession instead of simultaneously. We were led to adopt this plan by the consideration that arginase and urease have very different optima of pH. It seemed nevertheless worth while to determine whether Jansen's technique might not after all have some advantage in point of speed. To this end two sets of tubes were set up, each tube containing 12.0 mg. of arginine nitrogen in a solution buffered to a pH of 7.0. To the tubes of the first set were added 1 cc. of arginase and 1 cc. of urease, to those of the second arginase alone. At the end of 3 hours the contents of all the tubes were boiled, after which those of the second set were submitted to the action of urease. A subsequent determination of ammonia nitrogen gave for the first set an average of 1.63 mg. (27.2 per cent decomposition of the arginine), for the second 1.61. The presence of urease, at the reaction most favorable to it, had therefore not appreciably accelerated the action of the arginase.

*III. Use of Arginase in Determining the Arginine Content of Proteins.**A. Preliminary Considerations.*

The arginine content of proteins may be determined by applying the arginase method either directly to a neutralized (and preferably decolorized) protein hydrolysate, or to the "solution of the bases" obtained as part of Van Slyke's method of protein analysis (22), or to any other fraction of the hydrolysate in which the arginine may have been previously concentrated. If the object is to determine as accurately as possible the arginine content alone, the first of these applications constitutes undoubtedly the method of choice; the second has the possible merit of incorporating the arginase method in a well known wider scheme of protein analysis; the third has for the present hardly more than a theoretical interest. We have confined our attention therefore entirely to the first two.

Before either is discussed in detail it is necessary to take note of certain preliminary considerations, among the most important of which are those concerning the use of blank controls. It is in connection with these controls that we encountered the only special difficulty occasionally associated with the use of arginase in protein analysis.

1. Blank Controls for the Arginase-Urease Method.—In every case the gross result obtained by the use of arginase and urease must be corrected by deducting from it all ammonia other than that derived, through urea, from the arginine of the hydrolyzed protein. In dealing with an entire hydrolysate the total correction to be made includes then quite obviously (a) the ammonia fraction (or amide nitrogen) of the protein itself, (b) any ammonia present in the liver extract, (c) any ammonia in the urease, and (d) any ammonia produced by the urease from urea in the liver extract. The first of these items (a)—the "amide blank"—is to be determined on a separate sample of the hydrolysate; the other three, (b) + (c) + (d), are measurable at one operation by a single blank experiment—the "enzymes blank"—conducted with the enzymes alone. As a general rule the two controls thus indicated are all that are required. One complicating factor, however, does sometimes intervene. Some liver extracts—though not, in

our experience, more than two out of five—possess the property of liberating ammonia directly from some constituent or constituents of the hydrolysate; and this property, when present, adds of course to the total correction another item, (e) ammonia arising through the direct action of the liver extract upon the hydrolysate. Although, as has been intimated, this direct production of ammonia is the exception rather than the rule, it constitutes a possible source of error which one must always be prepared to encounter. It will be necessary therefore to consider in some detail its nature, its effect upon the determination of arginine, and the way in which that effect may be allowed for.

2. *Deaminizing Action of Certain Liver Extracts.*—The existence of an unexpected factor complicating the use of certain liver extracts upon protein hydrolysates was first noted in experiments of which the following is a typical example.

10 cc. of a gelatin hydrolysate, containing 48.20 mg. of nitrogen, were incubated with 1 cc. of a particular liver extract (Arginase 3) for 24 hours at 37° and pH 8.4. After treatment with urease the mixture yielded 5.61 mg. of ammonia nitrogen. The amide blank (a) was found to be 0.74 mg., the enzymes blank, (b) + (c) + (d), 0.34 mg. Deduction of both left 4.53 mg. apparently from arginine. The total arginine nitrogen of the sample appeared therefore to be $4.53 \times 2 = 9.06$, or, with the standard correction of + 1.5 per cent (see p. 641), 9.20 mg., which is 19.1 per cent of the total nitrogen. This was so far above any previous estimate that it could not possibly be a correct result.

The most obvious way of accounting for the excess ammonia of this experiment was to assume a deaminizing action upon the part of the liver extract. The correctness of such an assumption was demonstrated in the following way.

10 cc. of the same protein hydrolysate as before were treated with the same amount of liver extract and under the same conditions of time, temperature, and reaction. The ammonia was then determined *without any previous addition of urease*. The amount found, in terms of nitrogen, was 1.81 mg. This includes, of course, the items (a) and (b) of p. 642. The first (the amide blank) is already known to be 0.74 mg. The second, which may be called the "arginase blank," was found by a separate determination (actually carried out on 5 cc. of liver extract) to be

0.23 mg. Their sum is only 0.97. The mixture had therefore gained during incubation $1.81 \text{ minus } 0.97 = 0.84$ mg. of ammonia nitrogen, which must have arisen through the direct deaminizing action of the liver extract upon one or more of the amino acids present. This is item (e).

The total amount of ammonia, (a) + (b) + (e), found after incubating a hydrolysate with liver extract alone, might properly be called the "amide-arginase-deaminase blank," but for convenience we shall give it in future the briefer, if less fully descriptive, designation of the "deaminase blank."

In the experiment just recorded the deaminizing action found is not perhaps in itself of conspicuous importance; but it is sufficient to introduce a large error into the determination of arginine. If 0.84 mg. be deducted from 4.53, the result previously obtained for the apparent nitrogen from arginine, the latter is corrected to 3.69. On this basis the total arginine nitrogen is calculated to be only 7.49 mg., or 15.5 per cent of the total nitrogen. This is entirely consistent with the results of other methods of analysis (see Table XII).

3. Method of Correcting for Deaminizing Effect.—In calculating the above result we made use of as many as four blanks, but only three of these are really necessary. The total correction desired, (a) + (b) + (c) + (d) + (e), may obviously be found by adding the deaminase blank (a) + (b) + (e) to the enzymes blank (b) + (c) + (d) and deducting the arginase blank (b). The separate determination of amide nitrogen (a), unless required for other purposes, may be omitted. Even with three blanks the procedure may seem somewhat complicated; but it is less so in practice than in description, for neither the arginase blank nor the enzymes blank alters rapidly with time, and one careful determination of each may serve therefore for a whole series of analyses.

4. Action of Deaminizing Liver Extracts upon Different Fractions of the Hydrolysate.—It seemed worth while to ascertain whether the deaminizing action of certain extracts affected the whole amino acid mixture, or whether it was limited to any particular fraction thereof. With this object Arginase 3 (used in the preceding experiments) was allowed to act upon solutions of (1) glycocoll, (2) glutaminic acid, (3) arginine hydrochloride, (4) the bases

precipitated from gelatin by phosphotungstic acid, (5) the bases of casein, (6) the filtrate from the bases of gelatin as prepared in Van Slyke's method for nitrogen distribution, and (7) the mono-amino acids extracted from gelatin by butyl alcohol. In each case 5 cc. of the substrate, containing the quantity of nitrogen indicated in Table IV, were treated with 2 cc. of 0.25 M phosphate

TABLE IV.
Direct Action of Several Liver Extracts upon Various Substrates.

Arginase No.	Substrate.	Total N of sub- strate.	Ammonia N.				Deaminis- ing effect as per cent of total substrate N.
			Found.			Calcu- lated to result from deami- nation (e).	
			Total after incuba- tion (a) + (b) + (c).	In sub- strate (a).	In extract (b).		
		mg.	mg.	mg.	mg.	mg.	
3	Glycocoll.	28.18	0.18	0.04	0.13	0.01	0.03
3	Glutaminic acid.	23.70	0.21	0.02	0.13	0.06	0.25
3	Arginine-HCl.	26.60	0.16	0.01	0.13	0.02	0.08
3	Bases of gelatin.	18.62	0.29	0.06	0.13	0.10	0.54
3	“ “ casein.	8.89	0.22	0.03	0.13	0.06	0.67
3	Filtrate from bases of gelatin.	15.26	0.35	0.05	0.13	0.17	1.10
3	Butyl-extracted amino acids of gelatin.	32.69	1.02	0.17	0.13	0.72	2.20
3*	“ “	32.69	0.89	0.22	0.13	0.54	1.65
4	“ “	32.69	0.24	0.22	0.06	-0.04	-0.12
5	“ “	32.69	0.29	0.22	0.07	0.00	0.00
3	Total gelatin Hy- drolysate I.	48.20	1.81	0.74	0.23	0.84	1.74
6	Total gelatin Hy- drolysate II.	55.01	2.38	2.13	0.23	0.02	0.04

* After an interval of 11 months.

buffer of pH 8.4 and 1 cc. of liver extract, and were incubated at 37° for 24 hours. The total ammonia present was then determined in the usual way. At the same time we determined the ammonia originally existing as such not only in the extract, but also in each of the substrates. All these determinations were made in triplicate, though the average results alone are recorded

in Table IV. Table IV includes also (1) some results obtained 11 months later with the seventh substrate, in which the action of Arginase 3 is compared with that of two others (Arginases 4 and 5), and (2) the results of two similar experiments (the first of which has been described already) made with Arginases 3 and 6 upon unfractionated gelatin hydrolysates.

The last two columns of Table IV make it appear that of the two monoamino acids tested singly, one only (glutaminic acid) showed even a doubtful effect. Nevertheless it was evidently upon some constituent or constituents of the whole monoamino acid mixture that Arginase 3 exerted its main deaminizing influence. The mixed bases gave a result so small as to be almost negligible;⁶ arginine in particular was totally unaffected. It follows that the error introduced by the presence of deaminase is most important when the arginase method is applied directly to the total protein hydrolysate; in the analysis of the solution of the bases it may even, without great sacrifice of accuracy, be neglected.

5. Detection of Deaminizing Property in Liver Extracts.—Before employing, for purposes of protein analysis, any particular liver extract, it is important to know whether or not it possesses the special property now under discussion. If it does, three separate blanks are necessary for its proper control; if it does not, two will suffice.

Perhaps the simplest way of testing the point is to apply the extract to a substrate of completely hydrolyzed gelatin, or of butyl-extracted monoamino acids, in the manner exemplified by the experiment described on p. 643, or by those recorded in Table IV. If in such experiments the total amount of ammonia found after incubation is notably in excess of that originally present in extract and substrate, then deamination has taken place; otherwise, the extract is in this respect inactive. The last five experiments in Table IV offer examples of this test as applied to four different extracts. Three of these (Arginases 4, 5, and 6) are seen to have exerted no deaminizing action at all.

⁶ Incidentally this shows that the effect in question is not to be attributed to histidase. This enzyme, lately discovered by Edlbacher (23), decomposes histidine with the liberation not of urea, but of ammonia. Apparently it was absent from the liver extract employed; but if it were present, its effect would be included in our "deaminase blank."

As has been stated, the presence of deaminases in liver extract appears to be the exception rather than the rule. It is not difficult therefore to escape the complication (trifling as it may be) which they introduce. If the first extract prepared gives, with the test just described, a positive result, it is almost certain that the outcome with the second will be negative. It will be rarely indeed that one need prepare as many as three extracts before finding one that is free from deaminase. Such, at any rate, is the conclusion to be drawn from our own experience.

6. *Conditions for the Hydrolysis of the Protein.*—According to Jansen the arginase method gives the highest, and therefore presumably the most correct, results, when the protein, of which the arginine content is to be determined, is boiled with acid for 5 hours only. With longer periods of hydrolysis the apparent arginine content becomes progressively smaller. The explanation tentatively offered is that the hot acid gradually racemizes the liberated base.

It seemed worth while to ascertain whether this effect was really large enough to demand special attention. A series of experiments was therefore made with a high grade commercial brand of gelatin. Samples of a particular lot, which we shall designate Gelatin I, were boiled, each with a 10-fold quantity of 20 per cent HCl, for 1, 3, 12, 18, 24, and 72 hours, and a seventh sample was boiled with concentrated HCl for 84 hours. The arginine content of each hydrolysate was then determined by the method described on p. 652. The arginase extract used was Arginase 3. The essential data and the calculated results of the different determinations made are shown in Table V.

The outcome of these experiments is a definite confirmation of Jansen's statement that a short period of hydrolysis gives the highest results. The entire arginine content of gelatin appears to be liberated (or at least rendered susceptible to arginase) by as little as 3 hours of boiling with 20 per cent HCl; continued boiling distinctly diminishes the yield. It follows that any hydrolysis conducted with the sole purpose of determining arginine had better be of relatively short duration. On the other hand the longer period necessary and sufficient for complete hydrolysis does not seem to involve too serious a loss. Our results, in spite of certain discrepancies, indicate clearly enough that not more

TABLE V.
Hydrolysis of Gelatin.

Hydrolysate No.	Hydrolysing agent.	Time of hydrolysis.	Total N of sample.	Arginase 3.		Gross result of arginine determination.	Correction for blanks.*	Urea N from arginine.	Total arginine N (corrected†).	
				Quantity used.	Time of action.					
		hrs.	mg.	cc.	hrs.	mg. N	mg. N	mg.	mg.	per cent of total N
1	Boiling 20 per cent HCl.	1	48.47	1.0	24	4.70	1.57	3.13	6.35	13.10
			48.47	1.0	24	4.74	1.57	3.17	6.43	13.27
										13.19
2	Same.	3	45.46	1.0	24	5.27	1.72	3.55	7.21	15.85
			45.46	1.0	24	5.25	1.72	3.53	7.17	15.77
			45.46	1.0	24	5.30	1.72	3.58	7.27	15.99
			45.46	1.0	24	5.29	1.72	3.57	7.25	15.94
			27.27	0.5	24	2.91	0.80	2.11	4.28	15.69
			27.27	0.5	48	3.16	1.03	2.13	4.32	15.84
										15.85
3	"	12	78.02	1.0	24	7.47	1.47	6.00	12.18	15.61
			78.02	1.0	48	7.59	1.53	6.06	12.30	15.76
										15.68
4	"	18	24.10	1.0	12	3.05	1.19	1.86	3.78	15.69
			24.10	1.0	12	3.02	1.19	1.83	3.71	15.40
			24.10	1.0	24	3.28	1.43	1.85	3.76	15.60
			24.10	1.0	24	3.26	1.43	1.83	3.71	15.40
			48.20	1.0	24	5.61	1.92	3.69	7.49	15.54
										15.53
5	"	24	72.80	0.5	36	7.04	1.40	5.64	11.45	15.73
			58.30	0.5	18	5.63	1.10	4.53	9.20	15.78
										15.76
6	"	72	51.97	1.0	24	5.85	1.98	3.87	7.86	15.12
			51.97	1.0	24	5.83	1.98	3.85	7.82	15.05
			51.97	1.0	48	6.24	2.34	3.90	7.92	15.24
			51.97	1.0	48	6.18	2.34	3.84	7.80	15.01
										15.11
7	Boiling 7 concentrated HCl.	84	30.36	0.5	24	3.55	1.36	2.19	4.45	14.66
			30.36	0.5	48	3.72	1.57	2.15	4.36	14.36
			50.60	1.0	24	5.27	1.68	3.59	7.29	14.40
			50.60	1.0	24	5.30	1.68	3.62	7.35	14.52
										14.49

* Including in every case a "deaminase blank," which in turn includes the ammonia nitrogen of the hydrolysate.

† Calculated by doubling the urea N and increasing the result by 1.5 per cent.

than 2 per cent (and possibly less) of the total arginine is destroyed (or becomes non-reactive) within 24 hours. For many purposes it will be more important to insure complete hydrolysis of the protein as a whole than to avoid so small an error in the determination of one amino acid.

The arginine deficit after prolonged hydrolysis may, as Jansen suggests, be due to racemization; but it is possible that there takes place also some destruction of arginine, as of other amino acids, by deamination. The actual occurrence of deamination in our gelatin series was shown by a steady increase of ammonia from 1.28 per cent of the total nitrogen after 1 hour of hydrolysis to 1.39 per cent after 3 hours, 1.54 after 18, 1.59 after 24, 2.31 after 72, and 2.75 after 84. Just how much arginine contributed to this increase, it is of course impossible to say.

With this preliminary discussion of technical details and difficulties we may proceed to a working description of the several forms which the enzyme method of determining arginine in proteins may, under different circumstances, assume. We shall describe first those procedures (each adapted to a particular set of conditions) in which the arginase is applied without previous separation of the basic amino acids.

B. Direct Determination of Arginine in Protein Hydrolysates.

1. *Without Previous Removal of Humin and Ammonia.*—The most direct procedure of all, but one which is applicable only to rather lightly pigmented hydrolysates, is the following.

A given weight, say 10 gm., of the protein to be analyzed is boiled for 5 to 24 hours with a 10- or 20-fold quantity of 20 per cent hydrochloric acid. The hydrolysate is concentrated *in vacuo* to a thick syrup, and as much as possible of the free acid is driven off. The residue is dissolved in water, brought nearly but not quite to neutrality by the addition of sodium hydroxide,⁷ and made up to a definite volume. This volume should, if possible, be such that the quantity to be taken for the determination (preferably 5 cc., and in no case more than 10) shall contain

⁷ While it is convenient, it is not necessary, to carry out the neutralization at this stage. One may, if it is preferred, leave the main hydrolysate acid, and neutralize subsequently the separate samples taken for determination of arginine.

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between 6 and 12 mg. of arginine nitrogen. The concentration necessary to meet this recommendation may vary, in terms of the original protein, from 3 to 15 per cent.

The diluted hydrolysate is filtered, and a suitable aliquot is taken for the determination of total nitrogen.

Subsequent operations differ somewhat according as the arginase extract to be used does or does not contain deaminase. The simpler case, of course, is that of its absence.

(a) *When Deaminase Is Absent.*—Four 5 cc. portions of the prepared hydrolysate are pipetted into four Van Slyke-Cullen urea tubes. Two of these are reserved for the determination of the ammonia nitrogen of the protein—the amide blank. To each of the other pair (the “principal” tubes) are added successively 2 cc. of a 0.25 M solution of Na_2HPO_4 , a drop of phenolphthalein indicator, as much N NaOH (dropped from a burette) as is necessary to give a permanent light pink color,⁸ 1 cc. of an active arginase extract (such as described on p. 632) and a few drops of toluene. The two principal tubes are then covered with tin-foil, placed in a water bath at 37°, and left there for 12 to 24 hours. At the end of this period there are added to each 1 to 2 drops of phenol red solution and, dropwise, enough N HCl just to discharge the pink color. Meanwhile in each of a third pair of tubes—to supply the enzymes blank—there have been placed 3 cc. of water and 1 cc. of arginase extract. All six tubes having then been set up for aeration, 1 cc. of urease solution is added to the principal tubes and to the enzymes controls, but not of course to the remaining two. After the interval allowed for the action of the urease, the determinations are completed in the manner, and with the precautions, already set forth on p. 635. With the principal tubes, in which the volume of liquid will be rather large, it is well to use at least 9 cc. of saturated K_2CO_3 solution for the liberation of the ammonia, while 10 cc. of N/14 HCl (or 50 cc. of N/70) will suffice for its reception. With the other tubes each of these quantities may be reduced to 5 cc.

⁸ If the hydrolysate has not already been neutralized, it will be necessary, in order to keep the volume down, to commence this titration with a few drops of a strong (40 per cent) solution of alkali. At the same time the two samples reserved for the amide blank should be brought to a faint acidity to phenol red.

To illustrate the results obtained by this procedure we submit the protocol of an experiment made with Gelatin II, a sample of the same brand as Gelatin I but from a different lot. In this experiment most of the determinations were made not merely in duplicate, as recommended above, but in triplicate. The whole process, moreover, was carried through twice: once with Arginase 6, and again with a preparation, Arginase 7, which had been obtained from Arginase 6 by treating it, in an attempt at purification, with alumina cream. Since this treatment involved dilution by half, we employed Arginase 7 in double the usual quantity.

20 gm. of Gelatin II were boiled for 24 hours with 200 cc. of 20 per cent HCl, and the product was made up to a volume of 300 cc.

TABLE VI.
Direct Determination of Arginine in Gelatin II.

	With Arginase 6.	With Arginase 7.
	mg. N	mg. N
Gross result.....	6.800	6.782
Amide blank.....	2.132	2.132
Enzymes "	0.460	0.440
Total "	2.592	2.572
Urea from arginine.....	4.208	4.210
Total arginine found.....	8.416	8.420
" " corrected by 1.5 per cent.....	8.542	8.546
" N of substrate.....	55.02	55.02
	per cent	per cent
Relation of arginine N to total N.....	15.53	15.53

Total N.—3.013 cc. gave 33.13 and 33.17 cc. (average 33.15) N/14 NH₃; 5 cc. therefore contained 55.02 mg. of nitrogen.

Arginine, Gross Result.—5 cc., incubated for 23 hours, gave with Arginase 6, 34.06, 34.00, and 33.95 cc. (average 34.00) N/70 NH₃; with Arginase 7, 33.88, 33.91, and 33.94 cc. (average 33.91).

Amide Blank.—5 cc. gave 10.66, 10.64, and 10.67 cc. (average 10.66) N/70 NH₃.

Enzymes blank gave with Arginase 6, 2.29, 2.33, and 2.28 cc. (average 2.30) N/70 NH₃; with Arginase 7, 2.18 and 2.22 cc. (average 2.20).

In Table VI the titration averages found have been converted into mg. of nitrogen, and in that form used to calculate the arginine nitrogen of the gelatin. The sum of the two blanks

deducted from the gross result gives the quantity of urea nitrogen derived from arginine, the total nitrogen of the latter being of course just twice as much. The final addition of 1.5 per cent to the value found has been justified in a previous section (p. 641). The identity of the two results in Table VI and the excellent agreement of triplicates in the separate titrations of the protocol show that the procedure followed does, at the very least, give consistent and reproducible results.

It may be noted that the amide nitrogen of Gelatin II, as here determined, was 3.87 per cent of the total nitrogen. This is an unusually high figure; but it was confirmed in another determination made by the standard method of Van Slyke (see p. 659).

(b) *When Deaminase Is Present.*—If a deaminizing arginase extract is to be used, the procedure just described must be modified by substituting for the simple amide blank a “deaminase blank” and by adding an “arginase blank” for the determination of the ammonia preexistent in the extract. The method then takes the following form.

Four 5 cc. portions of a nearly neutral hydrolysate, prepared as already described (p. 649), are treated in urea tubes with 2 cc. of 0.25 M Na_2HPO_4 . Phenolphthalein is added and the mixtures are titrated with N NaOH to a light pink color. To *each* of the four are then added 1 cc. of arginase and a little toluene, and all are incubated at 37° for an identical period of 12 to 24 hours. At the expiration of this interval each tube receives a drop or two of phenol red, and is titrated with N HCl to a pH of about 6.8. To two of the four—the principal tubes—is now added 1 cc. of urease; to the other two—serving as deaminase blanks—no such addition is made. After an interval sufficient for the action of the urease, the determination of ammonia is completed with all four in the usual way. At the same time (or earlier) the two blanks further required are also performed in duplicate. One of these is the enzymes blank, to be carried out here just as in the method previously described. The other is the arginase blank, in which some convenient quantity, say 5 cc., of the liver extract has its ammonia content determined by treatment with K_2CO_3 and aeration in the usual way.

To illustrate the procedure we give in Table VII the results (each an average of closely agreeing duplicates) obtained on using

Arginase 3 in the analysis of Gelatin I. A quantity of this gelatin was hydrolyzed by boiling for 18 hours with 20 per cent HCl, and the hydrolysate (Hydrolysate 4, Table V) was afterwards made up to a volume which represented a 3 per cent solution of the original material. On this hydrolysate, in 5 cc. portions, two complete determinations were carried out. In one of these the arginase was allowed to act for 12 hours only, in the other for 24. The arginase blank for 1 cc. (the amount used) was calculated from an actually determined blank of 1.15 mg. of N for 5 cc. The net

TABLE VII.

Direct Determination of Arginine in Gelatin I, Exemplifying Use of Deaminase Blank.

	After 12 hrs. action of Arginase 3.	After 24 hrs. action.
	mg. N	mg. N
Gross result.....	3.035	3.27
Deaminase blank.....	1.10*	1.34*
Enzymes "	0.32	0.32
Arginase "	0.23	0.23
Net total "	1.19	1.43
Urea from arginine.....	1.845	1.84
Total arginine found.....	3.69	3.68
" " corrected by 1.5 per cent.....	3.745	3.735
" N of substrate.....	24.10	24.10
	per cent	per cent
Relation of arginine N to total N.....	15.54	15.50

* Of which only 0.37 mg. (as ascertained by a separate experiment) was contributed by the amide nitrogen of the protein. This corresponds to 1.54 per cent of the total nitrogen, or less than half as much as in Gelatin II.

total blank was calculated as indicated on p. 644. From the identity of the two results it is evident that Arginase 3, in the proportion employed, was active enough completely to decompose the arginine in 12 hours.

Other results obtained in the same way with the same and other gelatin hydrolysates have been reported in Table V.

2. *After Removal of Humin and Ammonia.*—The procedures hitherto described serve admirably for the determination of arginine in proteins, such as gelatin, which do not yield much pigment on boiling with acids. But the hydrolysates of most

proteins are so loaded with melanin, that for the adjustment of their pH (an operation called for at two points of the process) the indicator method is quite unsuitable. Under such circumstances two alternative courses are available. Either the pH adjustments must be made electrometrically, or, what will in general be more convenient, the interfering pigment must be removed.

An excellent way of removing pigment is to treat the hydrolysate with an excess of lime, as in Van Slyke's method for determining amide nitrogen. This has the advantage over other possible methods, of being in itself the first step in a standard system of protein analysis, and of combining with clarification of the hydrolysate a removal, and if desired a determination, of its ammonia. Van Slyke's original technique, however, is unsuitable. It introduces into the hydrolysate a very considerable concentration of calcium ions, and these, as Edlbacher (24) has shown, have an inhibiting effect upon the action of arginase. The application of the enzyme to the filtrate from lime and humin, as usually obtained, gives therefore, as we have found, a result almost invariably much too low. Fortunately the difficulty is one easily surmounted. If, before the addition of lime, the hydrolysate is neutralized, or nearly neutralized, with sodium hydroxide, the amount of lime necessary to provide an adequate excess is so reduced that the concentration of calcium ions need no longer reach a dangerous level. The neutralization may be carried out, as we have already recommended, on the total hydrolysate, before it is made to a definite volume; or, alternatively, it may just precede the addition of lime to the aliquot chosen for that treatment. The arginase method takes then the following form, which is to be regarded as a general method applicable to all proteins.

The protein is hydrolyzed, and the hydrolysate is neutralized and made to a convenient volume in the manner already described on p. 649. A suitable aliquot, containing the equivalent of 3 to 5 gm. of protein, is then submitted to Van Slyke's (22) procedure for the determination of ammonia, but with this special precaution, that only 5 cc. of 10 per cent lime suspension are used in making the mixture alkaline.⁹ When the distillation is ended, the residue

⁹ If the hydrolysate has not already been neutralized, the aliquot in the distilling flask must be made just acid to phenolphthalein with 20 per cent NaOH before the addition of the lime.

in the flask is filtered from lime and humin, the filter and its contents are washed free from chlorides, and filtrate and washings,

TABLE VIII.
Preparation and Preliminary Treatment of Hydrolysates.

Protein.	Hydrolysate No.	Quantity hydrolysed.		Time of boiling.	Volume to which hydrolysate was made up.	For lime treatment.		Ammonia found.		Volume to which lime filtrate was made up for determination of arginine.
		gm.	cc.			Volume.	Total N.			
				hrs.	cc.	cc.	mg.	mg. N	per cent of total N	cc.
Gelatin I.	5	20	200	24	200	60	873.6	13.90	1.59	100
Casein.	1	30	200	25	300	30	443.7	48.28	10.88	50
						30	443.7	48.69	10.96	50
						30	443.7	48.16	10.85	50
	2	20	200	26	200	60	817.2	89.67	10.97	50
	3	10	200	26	100	60	804.6			100
Edestin.	1	15	100	24	150	30	487.2	52.58	10.84	100
						30	487.2	53.49	10.98	100
	2	3	60	28	100	50	238.0	26.53	11.14	50
Gliadin.	1	10	200	24	400	100	349.4	86.27	24.69	50
						100	349.4			50
Globin.	1	10	100	24	100	60	938.1	51.10	5.45	100
Fibrin.	2	10	200	24	100	30	375.6	32.63	8.66	50
	3	10	200	24	350	200	742.4	64.34	8.67	100
	4	10	200	24	350	100	376.6	18.97*	5.04*	100
Ovalbumin.	1	5	100	22	50	25	278.5	22.82*	8.2*	50
Peptone (Witte).	1	20	200	5	400	100	700.0			100
“ (Merck).	1	20	200	24	400	100	695.0			100

* These figures are too low, indicating that the distillation with lime was not completely effective. The blanks in the subsequent arginine determination were correspondingly high (see Table IX).

faintly acidified with hydrochloric or acetic acid, are first concentrated and then made up exactly to 50 cc. or other suitable volume. The volume here chosen will depend on the quantity of

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TABLE IX.

Determinations of Arginine in Humin- and Ammonia-Free Hydrolysates.

Protein.	Hydrolysate and Sample No. (see Table V).	Volume analyzed.	Total protein N corresponding.	Arginase extract used.			Gross result.	Correction for blanks.	Urea N from arginine.	Total arginine N (corrected average).	
				No.	Quantity.	Time of action.					
		cc.	mg.		cc.	hrs.	mg. N	mg. N	mg.	mg.	per cent of total N
Gelatin I.	5	5	43.68	3	0.5	10	3.76	0.37	3.39	6.88	15.75
		5	43.68	3	0.5	34	3.81	0.42	3.39		
Casein.	3	5	40.23	3	1.0	24	1.96	0.37	1.59	3.21	7.98
		5	40.23	3	1.0	24	1.96	0.37	1.59		
		5	40.23	3	1.0	36	1.96	0.39	1.57		
Edestin.	1b	5	24.36	3	1.0	12	3.72	0.45	3.27	6.59	27.0
		5	24.36	3	1.0	12	3.70	0.45	3.25		
		5	24.36	3	1.0	36	3.71	0.49	3.22		
		5	24.36	3	1.0	36	3.75	0.49	3.26		
	2	5	23.80	3	1.0	24	3.50	0.43	3.07	6.25	26.3
		5	23.80	3	1.0	24	3.51	0.43	3.08		
		5	23.80	3	1.0	24	3.52	0.43	3.09		
Gliadin.	1a	10	69.88	3	1.0	24	2.30	0.64	1.66	3.37	4.82
		10	69.88	3	1.0	24	2.30	0.64	1.66		
Fibrin.	2	5	37.56	3	1.0	12	3.02	0.34	2.68	5.40	14.38
		5	37.56	3	1.0	12	3.02	0.34	2.68		
		5	37.56	3	1.0	36	3.06	0.43	2.63		
		5	37.56	3	1.0	36	3.07	0.43	2.64		
	3	5	37.12	3	1.0	24	3.12	0.51	2.61	5.25	14.15
		5	37.12	3	1.0	24	3.08	0.51	2.57		
		5	37.12	3	1.0	48	3.19	0.60	2.59		
		5	37.12	3	1.0	48	3.17	0.60	2.57		
	4	10	37.66	3	1.0	24	4.62	1.95†	2.67	5.42	14.39
		10	37.66	3	1.0	24	4.62	1.95†	2.67		

* Calculated by doubling the average found for urea N and increasing the result by 1.5 per cent.

† These high figures are due to incomplete removal of ammonia from the hydrolysate.

TABLE IX—*Concluded.*

Protein.	Hydrolysate and Sample No. (see Table V).	Volume analysed.	Total protein N corresponding.	Arginase extract used.			Gross result.	Correction for blanks.	Urea N from arginine.	Total arginine N (corrected average*).	
				No.	Quantity.	Time of action.					
		cc.	mg.		cc.	hrs.	mg. N	mg. N	mg.	mg.	per cent of total N
Ovalbumin.	1	10	55.70	3	1.0	24	4.11	1.29†	2.82	5.77	10.36
		10	55.70	3	1.0	48	4.19	1.33†	2.86		
Peptone (Witte).	1	5	35.00	4	1.0	12	3.08	0.70	2.38	4.85	13.85
		5	35.00	4	1.0	20	3.10	0.70	2.40		
" (Merck).	1	5	34.75	4	1.0	12	2.56	0.49	2.07	4.23	12.18
		5	34.75	4	1.0	12	2.59	0.49	2.10		

protein represented and on its arginine content; and should be such that 5 (or at most 10) cc. will contain at least 3, and if possible as much as 6 to 12, mg. of arginine nitrogen. To the nearly colorless solution thus obtained the arginase method is now applied in one or other (as may be called for) of the two modifications already described. When the arginase extract used contains no deaminase, one simplification is perhaps permissible. Since the substrate has been freed from ammonia, the amide blank is now in theory superfluous. It must, however, be recognized that the distillation with lime may leave behind at least a trace of ammonia, so that the inclusion of an amide blank is a precaution which it is not always altogether safe to neglect.

The method outlined has been applied to gelatin (Gelatin I, Hydrolysate 5), to casein, edestin, and wheat gliadin as prepared in this laboratory by standard methods, to Merck's fibrin, ovalbumin, and peptone, and to Witte's peptone. The protocols of the various analyses made have been brought together, in condensed form, in Tables VIII and IX. In Table VIII are shown for each case the steps leading to the preparation of the final solution suitable for the application of arginase. Table IX records the data relating to the actual determination and the calculation of the result. To save space there are described in Table VIII a few additional hydrolyses not utilized in the present series of determinations, but to be referred to in a later section.

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It should be said that in the case of the two peptones the ammonia was liberated by the addition, not of lime suspension, but of 1 gm. of MgO.

A comparison of the result for Gelatin I in Table IX with that for the same hydrolysate (No. 5) in Table V shows that the lime treatment, in the modified form adopted, has no effect which interferes with the determination. This is all the more evident when it is observed that the present result was obtained with only 0.5 cc. of arginase acting for the short interval of 10 hours. With regard to the other proteins the only remark that will be offered at present is that the different determinations made with any one are sufficiently consistent among themselves.

C. Determination of Arginine in a Solution of Bases Precipitated by Phosphotungstic Acid.

In Van Slyke's system of protein analysis arginine is determined in a solution of the bases precipitated by phosphotungstic acid. The method used is to boil the solution for a specified time with strong alkali, by which treatment half of the arginine nitrogen is converted into ammonia. There are several drawbacks to this procedure; and it seemed worth while to ascertain whether it might not, without other disturbance of the general scheme of analysis, be replaced by the more specific arginase method.

Preliminary experiments with pure arginine showed that after precipitation with phosphotungstic acid and regeneration from the precipitate it assayed by the arginase method from 93.3 to 94.7 per cent of the original quantity. Correction for the solubility of the phosphotungstate (a correction required whenever Van Slyke's general method is followed) raised these values to 99.0 to 100.3 per cent, on the average to 99.6. Apparently therefore the operations by which the solution of the bases is obtained introduce nothing which interferes with the arginase method.

The solution of the bases is usually made up to 50 cc. Of this 5 or 10 cc. (according as the protein is rich or poor in arginine) should be sufficient for an arginine determination. To provide a duplicate, 20 cc. at the most will be required, leaving a surplus sufficient for the estimation of total and amino nitrogen and cystine. Neither an amide blank nor a deaminase blank should

here be necessary. The solution of the bases, if properly prepared, will contain no ammonia to begin with; and it has already been shown to develop only a negligible amount under the action of deaminizing liver extracts. At the same time it must be recognized that, if any trace of ammonia should escape the distillation of the hydrolysate with lime, it will be precipitated with the bases by phosphotungstic acid; and an amide (or deaminase) blank furnishes security against this or any similar accident. To make possible the inclusion of such a blank it will be necessary, if all determinations are to be made in duplicate, to restrict the portions taken for the main experiment and the blank to 5 cc. Alternatively, single estimations may be made with portions of 10 cc. each.

As an example of the general procedure and the results obtainable we may here describe a determination carried out upon a solution of the bases from Gelatin II.

50 cc. of the diluted hydrolysate described on p. 651, containing 550.2 mg. of nitrogen, were submitted to the modified lime treatment (as already outlined) for the removal and determination of ammonia. The ammonia obtained neutralized 21.11 cc. of $N/14$ acid, which corresponds to 21.11 mg. of nitrogen or 3.84 per cent of the total. This agrees with the result previously obtained by the aeration method (p. 652).

The ammonia-free filtrate from lime and humin was treated in the usual way, and the bases were precipitated with phosphotungstic acid in a volume of 200 cc. The solution of the bases obtained from the precipitate was brought to a volume of 50 cc. To this solution, taken in triplicate samples of 5 cc., there was applied the method already described (p. 650) for the direct determination of arginine in protein hydrolysates. The arginase preparation used was Extract 6, which had no deaminizing action (see Table IV). The results obtained were as follows:

Arginine, Gross Result.—5 cc. gave 22.49, 22.50, and 22.46 cc. (average 22.48) $N/70 NH_3$.

Amide Blank.—5 cc. gave 0.07, 0.14, and 0.14 cc. (average 0.12) $N/70 NH_3$.

Enzymes blank gave 2.29, 2.33, and 2.28 cc. (average 2.30) $N/70 NH_3$.

From these data the arginine content of Gelatin II may be calculated as in Table X.

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This analysis illustrates the point, that in dealing with the solution of the bases the amide blank, however useful as a check upon the technique, is not an indispensable part of it. Its value, in relation to the actual arginine content, was here so nearly negligible, that its omission would have had a hardly appreciable effect upon the result. There is perhaps a greater possibility of error in the relatively large correction which has to be introduced for the solubility of arginine phosphotungstate. According to Vickery and Leavenworth (25) the true value of this correction is very uncertain. Nevertheless the result reached in Table X

TABLE X.
Arginine Determination on a Solution of Bases from Gelatin II.

	<i>mg. N</i>
Gross result.....	4.496
Amide blank.....	0.024
Enzymes ".....	0.460
Total ".....	0.484
Urea from arginine....	4.012
Total arginine found.....	8.024
" " corrected by 1.5 per cent.....	8.144
Correction for solubility of phosphotungstate (22)....	0.32
Final result for arginine.....	8.464
Total protein N corresponding.....	55.02
	<i>per cent</i>
Relation of arginine N to total N.....	15.38

(15.38 per cent of the total nitrogen as arginine) corresponds quite closely with that (15.53 per cent) obtained by a direct determination on the original hydrolysate (see Table VI).

Similar determinations were made on solutions of the bases obtained from Gelatin I, casein, globin, edestin, and gliadin. Details concerning the preparation of hydrolysates of these proteins have been given already in Table VIII. The last column of Table VIII shows the volume to which each hydrolysate, or sample of a hydrolysate, was made up after the removal of its ammonia. Of that volume the amount taken, in each selected case, for treatment with phosphotungstic acid was 50 cc. (sometimes the whole, sometimes half of the entire quantity available). The bases were precipitated under the exact conditions prescribed

TABLE XI.
Arginine Determinations in Solutions of Bases.

Protein.	Hydrolyzate and Sample No. (see Table VIII).	Volume.	Total protein N corresponding.	Arginase extract used.			Gross result.	Correction for blanks.	Urea N from arginine.	Total arginine N (corrected averages).	
				No.	Quantity.	Time of action.					
		cc.	mg.		cc.	hrs.	mg. N	mg. N	mg.	mg.	per cent of total N
Gelatin I.	5	5	43.68	4	1	24	3.53	0.34†	3.19	6.80	15.57
		5	43.68	4	1	24	3.53	0.34†	3.19		
Casein.	1a	5	44.37	1‡	0.1	24	1.91	0.36§	1.55	3.51	7.91
		5	44.37	1‡	0.1	24	1.95	0.36§	1.59		
	1b	10	88.74	1‡	0.1	41	3.60	0.39§	3.21	7.10	8.00
		10	88.74	1‡	0.1	41	3.55	0.39§	3.16		
	1c	10	88.74	1‡	0.1	24	3.59	0.39§	3.20	7.09	7.99
		10	88.74	1‡	0.1	24	3.55	0.39§	3.16		
	2	10	81.72	2	2	24	3.18	0.34§	2.84	6.43	7.87
		10	81.72	2	2	24	3.20	0.34§	2.86		
	3	5	40.23	5	1	24	1.77	0.31†	1.46	3.30	8.20
		5	40.23	5	1	24	1.79	0.31†	1.48		
Edestin.	1a	5	24.36	4	1	24	3.24	0.19§	3.05	6.49	26.6
		5	24.36	4	1	24	3.22	0.19§	3.03		
	1b	5	24.36	5	1	24	3.37	0.32†	3.05		
		5	24.36	5	1	24	3.39	0.32†	3.07	6.51	26.7
	5	5	24.36	5	1	24	3.36	0.32†	3.04		
		5	24.36	5	1	24	3.36	0.32†	3.04		
Gliadin.	1b	10	69.88	5	1	24	1.75	0.42†	1.33	3.34	4.78
Globin.	1	5	46.90	4	1	36	1.80	0.18§	1.62	3.62	7.72
		5	46.90	4	1	48	1.83	0.20§	1.63		
		5	46.90	4	1	48	1.83	0.20§	1.63		

* Calculated by doubling the average for urea N, increasing the result by 1.5 per cent, and adding (as a correction for the solubility of the phosphotungstate) either 0.32 or 0.64 mg., according as the volume taken for the determination was 5 or 10 cc.

† Including a deaminase blank.

‡ Arginase 1 was a dried preparation of the enzyme.

§ Enzymes blank only.

|| Arginase 2 was a 5 per cent filtered solution of Arginase 1.

by Van Slyke, and were brought again into solution in a uniform volume of 50 cc. The only instance in which this procedure was

modified was that of the second hydrolysis of casein; in this the bases were precipitated with 30 gm. of phosphotungstic acid in a volume of 400 cc., and their final solution was made up to 100 cc. In each solution of the bases thus obtained the arginine was determined by the application of the arginase method to duplicate or triplicate portions of 5 or 10 cc. The technical details and the results of these analyses are set forth in Table XI.

D. Summary of Results and Comparison with Those of Other Methods.

In Table XII we have collected (from Tables V to VII, IX, XI) the results of the arginase method (directly or indirectly applied) for each separate hydrolysate, or sample of a hydrolysate, analyzed, and have compared them with the results, obtained by ourselves or previously recorded by others, of three standard methods of arginine determination—the Van Slyke, the silver-baryta, and the more recent flavianic acid method of Kossel and Gross (26). All of the data, however expressed originally, have been brought of course to the same basis—percentage of total nitrogen in the form of arginine.

The different results obtained by the arginase method for any one protein show, for the most part, an excellent agreement. This is particularly true of the results by the direct method. Among six such reported for gelatin the extremes differ by only 2 per cent; and part of this difference, it has been shown, is due to differences in the time of hydrolysis. Results on solutions of the bases are apt, as with casein, to be rather less regular. The direct results are, of course, the more trustworthy; although, in a comparison of averages, there is little, if anything, to choose between them and the others.

The results of the Van Slyke method show considerable irregularity, but tend on the whole to exceed the arginase values. The data of the silver-baryta method are generally lower; those of the flavianic acid method more often higher. The discrepancies between the different estimates quoted are often, as with casein, very large. In most cases the arginase result lies, as might be expected of the true value, somewhere between the extremes.

In execution the arginase method is relatively simple. In principle it has the advantage over others of being strictly specific;

TABLE XII.

Per Cent of Total Protein Nitrogen in Arginine, As Estimated by Different Methods.

Protein.	Arginase method.			Van Slyke method.	Silver-baryta method.	Flavianic acid method.
	Hydrolysate No.	Direct determination.	Determination on solution of bases.			
Gelatin.....	2	15.85		14.23-15.26(22)	14.3-16.6(27)	16.45(26)
"	3	15.68		16.16*	15.87(26)	16.12*
"	4	15.53				
"	5	15.76				
"	5	15.75	15.57			
"	8	15.53	15.38			
		15.68	15.48			
Casein.....	1a		7.91	8.06(28)	7.51-7.83(28)	9.13(26)
"	1b		8.00	8.71-9.87(29)	7.8(30)	
"	1c		7.99	8.83-9.57*		
"	2		7.87			
"	3	7.98	8.20			
			7.99			
Edestin.....	1a		26.6	26.41-27.68(22)	24.7-25.4(31)	24.9(26)
"	1b	27.0	26.7	27.24*	27.25(25)	
"	2	26.3				
		26.7	26.7			
Gliadin.....	1a	4.82		5.64-5.80(22)	5.12(27)	
"	1b		4.78	5.29-5.62(32)		
				5.21*		
Globin.....	1		7.72	7.97-8.07(33)	6.48†(34)	
				8.19*		
				7.7†(22)		
Fibrin.....	2	14.38		13.21-14.32(22)		
"	3	14.15				
"	4	14.39				
		14.31				
Ovalbumin.....	1	10.36		11.7 (35)	10.2(36)	
Peptone (Witte)...	1	13.85			3.2(37)	
" (Merck)...	1	12.18				

Figures in parentheses refer to the bibliography.

* These results were obtained by ourselves on the same material as served for the arginase determinations.

† These figures are for hemoglobin.

and, since it has been shown to be truly quantitative, its results (if not vitiated by accidental error) deserve particular confidence. Our "direct" results for gelatin and fibrin, confirmed as they are by an adequate number of consistent determinations, constitute, we believe, as close approximations as any yet made to the true arginine contents of these proteins. Of the somewhat less concordant group of values yielded by edestin the highest is the most likely to be correct. The other results in Table XII can hardly be far from the truth, but may be allowed to require confirmation—those for casein because all but one were obtained indirectly, those for gliadin because they are so much below any previous estimate, the remainder because they are single determinations.

SUMMARY.

Methods are described for preparing and testing highly active solutions of arginase.

Under the action of the enzyme urease, urea has been found to yield 99.4 per cent of the theoretical amount of ammonia.

Arginase is capable of producing from arginine 99.1 per cent of the theoretical amount of urea.

By the successive use of the two enzymes, and with the application of a suitable correction, it is possible to determine arginine quantitatively with an error not exceeding 0.5 per cent.

Methods are described for the application of this procedure to the accurate determination of arginine in proteins.

Results are reported for the arginine content of gelatin and eight other proteins.

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THE DISTRIBUTION OF SERUM AND PLASMA PROTEINS IN FISH.

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INTRODUCTION.

Little is definitely known about the function of plasma proteins beyond the rôle played by fibrinogen in blood clotting and the rôle these proteins play in maintaining the osmotic pressure and viscosity of the blood. Of particular interest is the correlation of the albumin-globulin ratio with the edema of nephrosis. In this malady (1) the albumin-globulin ratio has been known to drop from its normal level of about 1.7 to 0.26. Moreover, it seems established that the serum albumin plays the predominating rôle in exerting the osmotic pressure of the plasma proteins (2).

Practically nothing is known about the plasma proteins of fish. It would seem that a study of the plasma proteins of fish should be of particular interest. The Elasmobranchii, for instance, are known to use urea which is present in their blood in very large amounts to maintain the osmotic pressure of their blood approximately equal to that of their environment (3). It would be of interest to determine whether this has any influence upon the concentration and distribution of their plasma proteins. Moreover, there are present so many varieties of fish in the sea, differing widely in activity, ecology, anatomical structure, and physiology, that it would not be too extravagant to look for differences in their plasma proteins with the possibility that information may be obtained which will ultimately throw more light on the function of plasma proteins.

An attempt was made to answer some of these questions in the summer of 1926 at the Bureau of Fisheries Laboratory¹ at Woods Hole. The data obtained there are presented in Table I.

¹ Acknowledgments are gratefully made to the United States Bureau of

Methods.

Howe's methods (4) for the fractionation of the plasma proteins were used. The menhaden and dogfish were taken as needed from a large floating live car and bled by cutting off the tail. Goosefish were bled with a hypodermic syringe from the heart or bulbous as soon as they were brought in from the traps. During the time that they were on the boat they were kept in large wooden tanks into which fresh sea water was flowing continually. Lithium oxalate was used as the anticoagulant, and the plasma was obtained by centrifuging. The globulins were fractionated with 14, 18, and 22 per cent sodium sulfate, and the albumin was fractionated with 26 per cent sodium sulfate. Since fibrinogen is precipitated by 10 per cent sodium sulfate, it was included in the euglobulin fraction. It was separately determined by precipitation with calcium chloride (5) and subtracted from the euglobulin fraction to obtain the euglobulins.

Nitrogen was determined by the Gunning-Arnold modification of the Kjeldahl method. 2 cc. of sulfuric acid were used, and the other reagents were correspondingly reduced.

Discussion of Data Obtained at Woods Hole.

Menhaden, Brevoortia tyrannus.—The total nitrogen in menhaden blood varies from 12.6 to 28.0 mg. per cc. It is difficult to state the cause of this variation. Asphyxiation is known to bring about changes in blood concentration (6) and with it great variations in blood total nitrogen, but the variation in the fish reported is not due to asphyxiation, as this factor was controlled. The plasma nitrogen varied from 1.9 to 5.1 mg. of N per cc. of plasma. Euglobulins were apparently entirely absent; only in Menhaden 3 (Table I) was there a significant amount. The albumin-globulin ratios were high, varying from 1.28 to 2.08.

Dogfish, Squalus acanthias.—The dogfish vary but little in the total nitrogen content of their blood and plasma. Euglobulins are always present in significant amounts. Their plasma differs strikingly from that of the menhaden in the great abundance of

Fisheries for defraying the expenses of that part of the investigation carried on at Woods Hole. The writer is also indebted to Mr. R. Goffin for help in collecting the fish used in the investigation at Woods Hole.

globulins as compared with the albumins, resulting in correspondingly low albumin-globulin ratios.

Goosefish, Lophius piscatorius.—The total nitrogen content of the blood of goosefish is low, but there is no great variation between the individual fish. From the hematocrit data it is evident that the low total nitrogen of goosefish blood is due to the low content of red blood cells. There is greater variation in the total plasma proteins. Euglobulins are always present in significant amounts. Like the dogfish, the plasma albumins are low as compared with the globulins, resulting in low albumin-globulin ratios.

Distribution of Plasma Proteins of Fish Caught in Monterey Bay.

The meager data obtained at Woods Hole left much to be desired. It seemed advisable to extend these studies. It was found impossible to do so at Woods Hole, so advantage was taken of the facilities at the Hopkins Marine Station² on Monterey Bay, Pacific Grove, California. In general, the same procedure was followed as in the Woods Hole studies, with minor changes to reduce the number of determinations for each analysis. Total nitrogen determinations of the blood were omitted. Serum was employed, thus eliminating the fibrinogen determination. The albumins were not fractionated, and the globulins were separated into euglobulin and pseudoglobulin only. Thus, many determinations were eliminated without omitting any very important fraction. Total plasma protein, euglobulin, pseudoglobulin, albumin, and non-protein nitrogen determinations were made. A slight modification suggested by Greenberg (7) was introduced. This consisted simply in the dilution of 1.0 cc. of serum with 19 cc. of sodium sulfate solution instead of the dilution used by Howe. The procedure is very convenient and equally accurate. Table II gives the data obtained on the fish in Monterey Bay.

² Thanks are due to Dr. L. B. Becking of the Jacques Loeb Laboratory at the Hopkins Marine Station for putting the facilities of his laboratory at the writer's disposal. My thanks are also due to Mr. MacGinitie for his cooperation in obtaining fish used in the analyses, and to Dr. H. M. Evans for permission to use apparatus belonging to the Department of Anatomy.

TABLE II.
Distribution of Serum Proteins of Fish from Monterey Bay.
 The results are expressed as mg. of N per cc. of serum.

	Dogfish No.							Bullhead No.				
	1	2	3	4	5	6	7	1	2	3	4	5
Plasma protein N.....	3.96	4.31	3.78	3.52	4.65	4.60	4.35	6.73	7.66	6.85	6.30	7.48
Euglobulin N.....	0.49	0.35	0.40	1.00	0.20	0.50	0.42	0.58	0.48	0.69	0.59	1.08
Pseudoglobulin N.....	2.35	2.88	2.55	1.62	3.60	2.85	2.78	4.40	4.88	4.31	4.56	5.00
Total globulin	2.84	3.23	2.95	2.62	3.80	3.35	3.20	3.98	5.36	5.00	5.15	6.08
Albumin N	1.12	1.08	0.83	0.90	0.85	1.25	1.15	1.45	1.90	1.45	1.87	1.21
Albumin:globulin ratio.....	0.39	0.33	0.28	0.34	0.22	0.37	0.36	0.29	0.35	0.29	0.36	0.20
Non-protein N.....	11.88	11.92	12.32	11.98	11.75	10.90	11.45	0.30	0.40	0.40	0.28	0.19

Discussion of Data Obtained from Monterey Bay.

Dogfish, Squalus acanthias.—The dogfish were caught in a drag net and bled by cutting off the tail as soon as they were brought on the boat. Some were more or less asphyxiated when taken from the net because of the large haul made. In no case did any of the blood clot. The samples were centrifuged, and the clear serum used for analysis. In general, the plasma proteins of the dogfish from Monterey Bay agree with those investigated at Woods Hole. The globulins greatly predominate over the albumins, resulting in low albumin-globulin ratios.

Bullhead, Scorpœnichtys marmoratus.—The bullheads were caught with a hook and line from the rocks on the shore and bled as soon as possible. Their blood formed loose but definite clots, and yielded serum colored green with a blue or purple fluorescence. The total plasma proteins were very high compared to those of the fish already investigated, and ranged from 6.3 to 7.6 mg. of N per cc. The globulins greatly predominate, again yielding low albumin-globulin ratios.

DISCUSSION AND SUMMARY.

Data on the distribution of the plasma and serum proteins of the blood of fish obtained at Woods Hole and Monterey Bay are presented. Different kinds of fish vary sharply in the total plasma proteins and in the distribution of the various fractions. From the data here presented it is impossible to explain these differences. Further investigation should throw light on these differences and make possible their correlation with the peculiarities of the fish. Should this be possible, it may throw important light on the function of the plasma proteins.

The elasmobranch, dogfish, in common with the decidedly unrelated teleosts, the bullheads and goosefish, have low albumin-globulin ratios. The bulk of the globulins is present in the pseudoglobulin fraction. It might be mentioned here that immune bodies are associated with the pseudoglobulins. Of these fish, the goosefish has the lowest total plasma protein content and the bullhead by far the highest, so, while these different fish have in common low albumin-globulin ratios, they differ greatly in their content of total plasma proteins.

The menhaden are characterized by their high albumin-globulin ratios.

Euglobulins are low in all the fish studied and absent in some of the menhaden.

The formed elements, mostly red blood cells, are significantly lower in the goosfish than in the menhaden or dogfish, and this accounts for the lower total nitrogen content of goosfish blood.

The fish from Monterey Bay are interesting from the standpoint of blood clotting. The dogfish blood did not clot at all, while the bullhead blood formed a loose but definite clot.

There are greater variations in plasma proteins among the teleosts themselves than between some of the teleosts and the elasmobranch, dogfish.

There is no significant difference between the plasma proteins of dogfish caught at Woods Hole, Massachusetts, and in Monterey Bay, California.

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STUDIES IN HYPERTHERMIA INDUCED BY THE HIGH FREQUENCY ELECTRIC CURRENT.

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INTRODUCTION.

This paper is concerned with the study of the blood chemistry following a rise in the body temperature of man induced by means of a high frequency electric current. Bazett and Haldane (1), Koehler (2), and Landis *et al.* (3) raised the body temperature by immersion in hot water. Cajori *et al.* (4) used the electric "bake," in which the subject is surrounded by an atmosphere of warmed air. In our studies the heat was generated within the subject's body, and the rise in temperature was at a much slower rate than that induced by hot baths. This eliminated the effect brought about by the rapid rise in temperature. Moreover the experiment was extended over a longer period of time, from $1\frac{1}{2}$ to $3\frac{1}{2}$ hours, in contrast to 30 minutes immersion in the hot bath. In our experiments the average rise in oral temperature was 0.01 – 0.015° per minute. In the experiments of Koehler and of Bazett and Haldane the rate was 5 to 10 times as fast. In Cajori's series the rate was almost exactly the same as in our series, but the highest temperature reached was only 38.4° . Walinski (5) has recently used a means similar to ours of raising the body temperature, apparently confining his analytical investigation to the determination of the CO_2 -combining power. He reports a fall in alkali reserve for the temperature range we studied. His data and method of arriving at this deduction are not given. It is possible that he confused CO_2 content with alkali reserve.

We were particularly interested in determining the plasma pH directly by means of the quinhydrone electrode and in studying the inorganic phosphorus changes.

Plan.

As originally planned, the subject was to fast 12 hours before the heating. Two of the individuals who followed this schedule suffered with severe headaches after the experiment, one of them being nauseated. The remaining individuals who were studied had eaten 6 hours before the heating was begun. Two cancer patients, who were heated in addition to the six normal individuals, had fasted 12 hours. 24 hour urine specimens were collected up to the time of heating. Another urine specimen was collected to cover the time of heating and recovery period up to the point where the temperature was back to normal. Blood samples, taken from the arm vein, were collected before heating and when the maximum temperature had been reached. In two instances part of the initial blood sample was lost. These subjects were bled at the same time, on another day. For four of the subjects basal rates were taken before the heating, and at the time of the maximum rise in body temperature.

Total CO₂, oxygen capacity, and combined oxygen were determined by the Van Slyke and Neill manometric methods. Inorganic phosphorus was determined by the Fiske-Subbarow modification of the methods of Bell and Doisy and Briggs. The blood was precipitated within 5 minutes after being drawn. Calcium was determined by the Clark-Collip modification of the Kramer-Tisdall method. The pH of the plasma was determined by the quinhydrone electrode, with the technique recommended by Cullen. The values in the normal range were reproducible to 0.01 pH. The electrode potential drift for the two very alkaline pH values reported was greater than for the normal values, so that these values were not reproducible to more than 0.02 pH. The pH values are reported at the oral temperature of the patient, when the blood was sampled and not at 38°, as is done conventionally. The calculation is based upon the relation used by Cullen and others, $\text{pH}_t = \text{pH}_i - (0.01 \times [t_i - t])$.

The machine used for heating was one made especially for this work by one of the x-ray manufacturers. It was designed to give

considerably greater power than the ordinary diathermy machine in general use, and was also designed to give an especially smooth high frequency wave to prevent the subject from suffering any disagreeable sensations due to passage of current. With the exception of the first experiment in which the electrodes were smaller, large lead electrodes covering the major portion of the back and chest were used. In short the machine was designed to enable us to apply to the subject a maximum of power with a minimum of discomfort.

Results.

Plasma pH.—For the seven individuals studied the plasma pH rose in every case. The maximum rise was from 7.47 to 7.70 pH, a change of 0.23. Three individuals showed a rise of 0.06 pH. Three others fell within these ranges. It should be noted that these differences would be from 0.02 to 0.03 pH greater if the values were reported at 38° instead of at the oral temperature. In every instance the pH rose above the normal range of 7.40 to 7.50 as established by Cullen and Earle (6) for the quinhydrone electrode. Up to date we have had occasion to determine the pH of fifteen apparently normal individuals, using Cullen and Earle's technique with slight modifications. This group fell within the range 7.42 to 7.50 pH (38°). In every case therefore the rise in body temperature raised the plasma pH above the normal limits. In two cases the pH was only 7.52 and 7.53. It will be noted that one of the cancer patients had an initial pH of 7.54, 0.04 above the upper limit of normal. Although this man's temperature was only raised 0.22° the pH rose 0.10. The other cancer patient, having a subnormal temperature of 36.6° initially, was heated to only 37.2°. The pH changed 0.08. Although these temperatures were only slightly above normal, the heating had to be discontinued because of the marked distress of the subjects. Excluding the cancer patients, who showed very weak powers of adjustment to changes in their body temperature, the two normal individuals who manifested the greatest hyperpnea showed the greatest change in blood pH. The pH data will be found in Table I.

Alkali Reserve.—In all of our heating experiments the total CO₂ content fell from 4 to 12 volumes per cent. Koehler (2) observed a similar change due to rise of body temperature by hot baths, as

TABLE I.
Changes in Blood and Urine, Following Rise in Body Temperature.

Name.	Date and time.	Temperature.		Plasma.		Blood.			Urine.			
		Oral temperature.	pH at body temperature.	TCO ₂	Ca	THb	HbO ₂ THb	P	24 hr. P.	P heating period.	24 hr. pH.	pH heating period.
		°C.		vol. per cent	mg. per 100 cc.	vol. per cent	per cent	mg. per 100 cc.	gm.	gm.		
H. J. U.	4-5-29											
	7.15 p.m.	37.0	7.47	63.5	9.95	21.5	67	3.6				
	10.45 "	39.4	7.70	51.9	10.9		98	2.1				7.2
	4-6-29											
	7.10 a.m.			64.5				3.3				
F. E. B.	4-16-29											
	8.00 a.m.	36.7	7.46	68.2	9.06	23.4	46	3.1	1.08		6.2	
	4-17-29											
	11.30 a.m.	39.0	7.52	60.9	9.59	23.0	77	2.1		0.01		7.7
	1.30 p.m.	37.0	7.52	60.9	9.87	23.2	80	2.5				
[E. C. W.	10-4-29											
	8.00 a.m.	37.4	7.48		10.3			3.4			6.0	
	9.30 "	38.7	7.68	43.9	11.0	24.0	100	2.8		0.008		7.9
J. E.	5-14-29											
	10.00 a.m.	37.3	7.54	63.5	9.63	16.7	63.5	3.2				
	11.00 "	37.5	7.64	53.3	9.95	16.9	97	2.7				
A. G.	5-30-29											
	10.00 a.m.	36.6	7.48	66.0	9.44	18.1	67	3.0				
	11.30 "	37.2	7.56	60.1	9.77	17.8	96	4.0				
M. L. L.	10-17-29											
	7.00 p.m.	37.4	7.47	60.4	9.7	17.8	52	3.2	0.65		6.0	
	8.40 "	38.9	7.53	56.0	9.9	17.4	56	3.3		0.088		5.8
Z. T.	10-17-29											
	7.00 p.m.	37.5	7.49	54.3	9.8	20.6	79	3.3	0.54		4.8	
	10.20 "	39.2	7.55	50.8	9.7	21.4	95	2.6		0.003		6.0
E. M. O.	10-15-29											
	7.35 p.m.	37.1			11.0			3.0	1.11		6.2	
	9.10 "	38.7			12.3			2.7		0.065		5.8

TCO₂ indicates the total CO₂, the sum of the combined and dissolved CO₂. THb indicates the total hemoglobin as measured by the oxygen-combining power and expressed as volumes per cent of oxygen. HbO₂ indicates the oxyhemoglobin. P in the columns under blood and under urine indicates inorganic phosphorus.

did Cajori *et al.* (4) in experiments with electric "bakes." Since the pH of the plasma and oxygenation of the hemoglobin have both increased, one is not justified in regarding the condition as one of lowered alkali reserve. Cajori *et al.* determined the CO_2 absorption curves for their blood specimens after heating and compared the BHCO_2 content at the initial pH. Their results showed a slight rise in the alkali reserve.

We have calculated the increase in base bound by the blood proteins during heating, using the constants worked out for horse hemoglobin, globulin, and albumin by Van Slyke *et al.* (7, 8). For hemoglobin and oxyhemoglobin our experimental values are used. For globulin and albumin we have assumed a normal value of 1.0 gm. of nitrogen per 100 cc. of blood.

$$\text{BHb} = 2.6 (\text{Hb}) (\text{pH} - 6.81) + \text{HbO}_2 \left(\frac{1}{1 + 10^{6.87 - \text{pH}}} - \frac{1}{1 + 10^{8.33 - \text{pH}}} \right)$$

$$\text{BP}_p = 0.66 \times \text{gm. N} \times (\text{pH}_p - 5.08)$$

These calculations can of course be considered only semiquantitatively, since the constants used are not for human blood. For globulin and albumin Van Slyke *et al.* have shown that the base bound by the proteins of human serum agreed within 6 per cent with the amounts calculated. The results are summarized in Table II and indicate no change or a slight increase in the alkali reserve of the blood. The last column in Table II, $\Sigma\Delta\text{B}$, represents the net change in the base held by the blood, and is the algebraic sum of the results of the first three columns. The increase in base bound by the phosphates has not been calculated, as the amount is small when compared with the hemoglobin and bicarbonate.

Phosphorus.—Lawaczek (9) and Jost (10) have shown by *in vitro* experiments with whole blood that there is a reversible reaction between the organic and inorganic phosphorus, the two forms being interconvertible. Equilibration with CO_2 shifts the reaction so that organic phosphorus is hydrolyzed to inorganic phosphorus. Equilibration with air, or addition of bicarbonate, increases the organic phosphorus with diminution of the inorganic. In view of these observations the inorganic phosphorus of the whole blood was followed before and during heating in our experiments to determine whether the Lawaczek reaction took place *in vivo*. Since the pH

of the blood may be changed so markedly with rise of body temperature, the *in vitro* condition is duplicated in the body. Six individuals showed a fall of the inorganic phosphorus of the whole blood ranging from 10 to 42 per cent. One normal individual showed no change and one of the cancer patients showed a 25 per cent rise. The cancer patient, who had an extensive carcinoma of the prostate, had been treated with x-ray and lead; barring this case the data indicate that the conversion of blood inorganic to organic phosphorus takes place in the body with rise in pH. An examination of the data for the inorganic phosphorus excreted in the urine during the period of heating is illuminating. The individual who showed no change in blood phosphorus excreted the largest amount of urine phosphorus. The individual who showed

TABLE II.

Change in Distribution of Base, Bound by Buffers of Blood during Heating.

	$-\Delta\text{BHC}\text{O}_3$	$+\Delta\text{B}(\text{Hb})$	$+\Delta\text{BP}_g$	$\Sigma\Delta\text{B}^*$
	<i>mm per l.</i>	<i>mm per l.</i>	<i>mm per l.</i>	<i>mm per l.</i>
F. E. B.	2.9	4.2	0.5	+1.8
J. E.	4.2	3.7	0.7	+0.2
A. G.	2.6	3.4	0.5	+1.3
M. L. L.	2.1	1.6	0.5	+0.0
Z. T.	1.4	3.4	0.5	+2.5

* $\Sigma\Delta\text{B}$ represents the net change in base held by the blood.

a lowering of 10 per cent in the blood phosphorus showed the second largest urine phosphorus output; while the individuals who showed appreciable drops in blood phosphorus excreted only a few mg. of phosphorus in the urine. This observation is interesting as it indicates a renal threshold for phosphates. Collip (11) in his experiments on hyperpnea (by forced breathing) reported an increase in urine phosphates. Bazett and Haldane, while they report an alkaline urine during hyperpnea by hot baths, mention the presence of carbonate, but not phosphate. It must be remembered however that in our experiments the duration of time was 2 to 3 hours, while in the production of hyperpnea by forced breathing or hot baths the time element is considerably reduced.

Blood Calcium.—In the eight subjects studied the blood calcium rose 7 or more per cent over the initial value in four cases. This

change could not be attributed to reduction in blood volume as judged by the hemoglobin analysis. One is tempted to point out a relation between the fall in inorganic phosphorus and rise in calcium in harmony with the solubility product concept.

Oxygenation of Hemoglobin.—In all the cases studied the hemoglobin of the venous blood was more highly oxygenated following a rise in the body temperature. In five of the seven cases it was over 95 per cent saturated during the heating. In the other two cases, the per cent oxygenation was within normal limits.

Urine pH.—Urine samples for the six normal individuals were collected for the total period of heating. The pH values were 5.8, 6.0, 7.2, 7.7, and 7.9. The urine samples were neither collected nor kept under oil, so that one is not justified in considering the values 7.7 or 7.9 more alkaline than that of blood. Landis *et al.* (3) give pH values for urine excreted during hot baths, which fall within a range identical with ours. Unless they used special precautions to retain the CO₂ tension of the urine as drawn, they are not justified in concluding that the urine of three of their subjects was more alkaline than the blood. The three young women who took the heating so very easily showed acid urine, although their blood pH rose 0.06. The most alkaline urine specimens found by us were for the three subjects who had shown the most marked hyperpnea. In the one case, our first experiment, the body temperature had been raised too rapidly and the subject at one period gasped for air. The two other subjects had basal rates taken at their highest temperatures. The taking of the basal rate, as later mentioned, had a marked effect upon the respiration, resulting in air hunger.

Metabolic Rate.—In their experiments with hot baths Bazett and Haldane noted that the intensity of hyperpnea varied with the rate of rise rather than the temperature. With a temperature of 37.2° rising at 0.13° per minute, 27.3 liters per minute were breathed by one of their subjects against a normal of 6 liters. With a steady body temperature of 38.6°, 12.7 liters per minute were breathed. In one of our experiments the metabolic rate was also taken at 38.6°. During this period the body temperature was kept constant. 6.6 liters were breathed against a normal 5.2. For another subject the temperature rate was 0.03° per minute while the metabolic rate was being taken. In this case 17.0 liters were

breathed against a normal 4.3. The respiratory quotient rose to 1.08 (initial 0.83). Bazett and Haldane obtained a respiratory quotient of 1.30 when the temperature was raised rapidly. Koehler found an increase of volume respiration from 6.2 to 19.5 liters when the temperature was raised to 39.4° at the rate of 0.2° per minute. In this instance the respiratory quotient fell from 0.77 to 0.69. Landis *et al.* made a rather exhaustive study of the respiratory changes following hot baths. Our observations confirm the findings of these authors. The taking of the basal rate at the oral temperature of 39° had in itself a pronounced effect upon the subject. In each case the current was turned off, while the basal rate

TABLE III.

Respiration Data Taken at Initial Body Temperature and During Heating Experiment at Time of Maximum Rise in Body Temperature.

	Oral tempera- ture.	Respira- tion volume.	O ₂ consump- tion.	CO ₂ output.	R Q.	Pulse.	Respira- tion rate.
	°C.	l. per 10 min.	l. per 10 min.	l. per 10 min.			
F. E. B.	36 6	52.9	2 32	1 80	0 78	66	14
	38 6	66 9	2 99	2 09	0 70	112	20
E. C. W.	37.4	43.3	1 75	1 46	0.83	84	18
	38 7	170 2	3 25	3 52	1.08	104	28
J. E.	37.3	45.9	2 18	1 41	0 65	79	18
	37 4	77 5	2 63	1 95	0 74	86	28
A. G.	36 6	65 7	2 10	1.69	0 80	78	20
	37 5	76 2	2 59	2 03	0 79	82	24

was being taken, so that there would be no rapid rise in body temperature. In spite of this precaution the pulse and respiration rate rose during the collection of expired air. The subjects felt air hunger, one of them complaining of a tingling sensation in the arms and legs. The data must therefore at best represent an exaggerated picture. Du Bois (12) has shown that the basal metabolism of a fever patient is 13 per cent higher for each °C. above normal. For one of our normal subjects we virtually obtained this figure. His temperature rose from 37° to 38.8°, his basal metabolic rate from -9 to +16. The other normal subject, whose pulse rose from 100 to 120 and whose respiration rose from 20 to 28 during the taking of the rate, showed a net increase of 83 per cent in the rate.

Protocols.

Time.	T	P	R	Time.	T	P	R	Time.	T	P	R
F. E. B., male, Apr. 17, 1929.				A. G., male, May 31, 1929; cancer patient.				E. M. O., female, Oct. 15, 1929.			
<i>a. m.</i>				<i>a. m.</i>				<i>p. m.</i>			
9.00*	37.0	72	14	8.00*†				7.35†	37.1	92	16
9.15	37.0	70	18	10.00	36.6	76	20	8.00	37.1	88	15
9.30	37.1	72	20	10.15	36.7	68	20	8.15	37.5	98	16
9.45	37.2	78	20	10.30	36.9	72	20	8.30	37.8	90	18
10.00	37.4	84	20	10.45	37.1	80	20	8.45	38.3	110	16
10.15	37.7	86	20	11.00*	37.5	82	22	9.00	38.6	110	18
10.30	38.1	92	20	11.15		88	24	9.07†	38.7		
10.45	38.3	100	20	11.30†	37.2	90	20	9.14	38.3		
11.00*		104	24	<i>p. m.</i>				9.30	37.8		
11.15	38.9	120	20	4.00	36.5	80	20	10.00	37.4		
11.30†	39.0	116	22	E. C. W., female, Oct. 4, 1929.				Z. T., female, Oct. 17, 1929.			
11.45	39.1	108	20	<i>a. m.</i>				<i>p. m.</i>			
12.00	38.9	104	20	7.30*†				7.00†			
<i>p. m.</i>				8.00	37.4	80	18	8.50	37.5	100	24
12.15	38.5	90	20	8.15	37.4	72	20	9.05	37.6	100	24
12.30	38.2	92	20	8.30	37.5	80	20	9.15	37.7	112	24
12.45	37.8	84	20	8.45	37.7	104	20	9.30	38.3	120	28
1.15	37.1	76	20	9.00	38.4	100	20	9.37	38.6	137	32
2.15	36.7	84		9.15*	38.5	104	28	9.45	38.8	148	28
H. J. U., male, Apr. 5, 1929.				9.30†	38.7	120	28	10.00	39.0	160	28
<i>p. m.</i>				9.45	38.6	84	20	10.05	39.2	140	28
7.30†	37.2	84	18	10.15	37.5	88	20	10.17	39.2	140	28
8.00	37.0	76	18	11.15	37.5	84	20	10.20†			
8.15	37.2	84	20	11.45	37.2	84	20	10.25	39.2	140	28
8.30	37.4	92	24	J. E., male, May 14, 1929; cancer patient.				10.40	38.8	120	28
8.45	37.6	90	24	<i>a. m.</i>				11.00	37.7	120	28
9.00	37.9	96	20	8.00*†				11.45	37.2	100	22
9.15	38.2	99	24	10.00	37.3	88	20	M. L. L., female, Oct. 17, 1929.			
9.30	38.5	102	24	10.15	37.5	84	20	<i>p. m.</i>			
9.45	38.8	102	24	10.30	37.5	90	18	7.00†	37.4	72	24
10.00	39.1	102	24	10.45	37.5	86	20	7.15	37.3	72	24
10.15	39.3	104	24	11.00	37.2	100	32	7.30	37.5	88	20
10.30†	39.4	102	24	11.15†				7.45	37.8	96	24
10.45	39.2	100	24	11.30*	37.4	88	28	8.00	38.1	100	26
11.00	39.0	100	20	11.45	37.4	86	20	8.15	38.5	112	24
11.15	38.0	96	20	<i>p. m.</i>				8.30	38.9	112	24
11.30	37.6	94	20	4.00	37.0	84	20	8.35†	38.9		
11.45	37.1	92	20					8.55	38.8		
12.00	36.9	92	18					9.25	37.7	92	24
								10.10	37.2	80	20

Abbreviations used in protocol headings: T = oral temperature, °C.
P = pulse rate. R = respiration rate.

* Basal metabolic rate taken.

† Blood taken.

This subject as well as the cancer patients had completely oxygenated hemoglobin at the time, while the subject who responded less markedly showed 80 per cent saturation for his hemoglobin. His pulse also rose to 120 during the basal rate, but the respiration rate rose only from 20 to 24. The data discussed in this paragraph will be found in Table III. In the protocols are recorded the oral temperature, the pulse, and respiration rate during the course of the heating.

DISCUSSION.

The results show rather conclusively that there is no difference in effect between raising the body temperature by a high frequency electric current, so that the heat is generated within the body, and in raising the body temperature by the external application of heat.

Of all the changes taking place in the chemistry of the body following a gradual moderate rise in body temperature (37.0–39.4°) the one of fundamental importance is the loss of CO₂. This has been pointed out before by others. Our data illustrate this point very strikingly. The one effect which was the same in all the experiments was the rise in the blood pH. This, in connection with the other data, showed conclusively a lowered CO₂ tension of the blood. The other changes, which in some instances did not follow, were a result of the lowering of the CO₂ tension. Thus, the pH of the urine remained unchanged or became more alkaline, the alkali reserve remained the same or increased, the inorganic phosphorus was unchanged or decreased, the calcium was unchanged or increased, the hemoglobin was more or less highly oxygenated. The change in the total CO₂ content of the blood is readily accounted for by the shift of base to the blood proteins due to the increase in pH. Since the alkali reserve of the blood remains the same or increases slightly, and since the pH of the urine as excreted is not more alkaline than the blood, there is little reason for believing that the body is attempting to compensate for the lowered CO₂ tension by lowering the alkali reserve. Experiments with forced breathing and those in which the body temperature is raised very rapidly by immersion in hot water are not to be placed in this category. The very alkaline urine and fall in urine ammonia observed under these conditions indicate the attempt of the body to compensate.

The question of whether or not the body is attempting to lower

the alkali reserve when the CO_2 tension becomes lowered and the pH raised is of considerable importance clinically. If the body were trying to decrease the alkali reserve during a fever, in order to bring the pH back to normal, it would assuredly be contraindicative to the forcing of alkalinizing agents.

Cajori *et al.* do not agree with Koehler that there is an anoxemia in fever alkalosis. They found, as did we, that there is always an increase in the oxygen saturation of the venous blood following the increase in pH of the blood. It does not appear to us that the question can be settled with the data available up to date. There are too many factors involved. As a result of the lowered CO_2 tension of the blood, and increased pH, the stability of the oxyhemoglobin increases. If the circulation and metabolism did not increase at the same time, the question would be quite simple. With the increase in circulation, however, the tissues are exposed to more blood per unit of time, so that the effect of the stability of the hemoglobin might be offset if the demand for more oxygen due to increased metabolism were not too great.

A study of the nature reported in this paper is only possible with the cooperation of a large number of people. We are especially indebted to Mr. Carl Darnell, of the Victor x-Ray Corporation, to Miss Ella M. Ottery, and to Mr. L. C. Maxwell.

SUMMARY.

There is no difference in effect between raising the body temperature by a high frequency electric current so that the heat is generated within the body, and in raising the body temperature by the external application of heat. Of fundamental importance is the loss of CO_2 . The pH of the blood becomes more alkaline. There is a shift of bases to the blood proteins. The hemoglobin of venous blood is more oxygenated.

With rise in blood pH the inorganic phosphorus was observed to fall below normal limits in some cases. This observation is in harmony with Lawaczek's *in vitro* conversion of inorganic to organic phosphorus in blood. The urine phosphorus was markedly decreased during the period when the blood phosphorus fell.

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THE EFFECT OF VITAMIN D AND OF REACTION OF DIET UPON RESPONSE TO PARATHYROID EXTRACT.

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A relation of the antirachitic vitamin to the function of the parathyroid glands has recently been suggested by several investigators. The prevention or postponement of tetany in parathyroidectomized dogs by cod liver oil or irradiated ergosterol fed before or after the operation was reported by Jones (1), Brougher (2), and Wade (3). Greenwald and Gross (4) on the other hand found very little protection afforded such animals, even by massive doses of irradiated ergosterol. The latter authors offer the explanation that residual parathyroid tissue may be so stimulated by the antirachitic vitamin as to delay the fall in concentration of serum calcium until the remaining and accessory tissue has hypertrophied sufficiently to supply the animal's needs. The level of calcium in the diets used both before and after the operation and the need for calcium of the animal as modified by growth or lactation are suggested as important modifying factors in the degree of protection afforded by the vitamin.

Several other substances have been found to prevent the tetany of parathyroidectomized animals. Among these have been listed milk, lactose, calcium lactate, acetate, or carbonate, and ammonium chloride by mouth, or the potent parathyroid extract of Collip by subcutaneous injection. The mechanism by which these diverse agencies prevent tetany, as well as the mode of production of tetany in the absence of the glands, remains to be satisfactorily demonstrated. In view of the recent thorough review of the evidence on these points by Greenwald (5) and Greenwald and Gross (4, 6), no attempt will be made here to

summarize the theories offered to explain the facts which have been observed.

It is obvious, however, that a profound influence is exerted by the parathyroid glands upon the calcium-phosphorus metabolism, as is shown by the drop in level of serum calcium in parathyroidectomized animals, and the corresponding rise observed in animals to which the parathyroid extract has been administered. The retention of calcium and phosphorus in the former animals and their increased excretion in the latter are generally noted (7).

All calcium and phosphorus balances made upon either parathyroidectomized or parathyroid extract-treated animals have so far dealt with adults and usually adult animals of varied and often unknown early history. Practically all the blood sera studies of Collip *et al.* (8, 9), Salvesen (10), and Reed (11) were made upon adult dogs, sometimes specified as young adults, but without definite age data. Such animals are presumably well supplied with body stores of excess vitamin D, and are ordinarily not sensitive to the administration of excess or to short intervals of deficit in intake of that vitamin. Crucial tests therefore of the interrelation of the antirachitic vitamin and the parathyroid secretion can scarcely be said to have been made.

In *young* animals the administration of cod liver oil, of irradiated ergosterol, or of direct ultra-violet irradiation has been shown by numerous workers to raise the level of blood calcium and inorganic phosphate, to relieve tetany, and to heal rachitic lesions. Whether such remedial action is possible in the absence of normally functioning parathyroids is a question raised by the recent work of Hess, Lewis, and Rivkin (12), who found that in a parathyroidectomized monkey irradiated ergosterol was impotent to raise the level of serum calcium, although the same animal had previous to operation responded by a rise in serum calcium to the normal figure from the low level induced by calcium-deficient diet. Apparently animals in active rickets have not been used for parathyroidectomy nor for observation of the effects of injection of parathyroid extract.

Nevertheless a relationship between diseases characterized by defective calcification of bones and teeth and abnormality of the parathyroid glands has frequently been pointed out. Erdheim (13) found defective enamel in the teeth of parathyroidectomized

rats and recently Jung and Skillen (14) have described similar changes. Hypertrophy or tumor of the parathyroids has been observed by Bauer (15) and by Ritter (16) in osteomalacia, and tumors of the glands were found in one case of osteitis fibrosa by Dawson and Struthers (17) and in another by Lambie (18). Grant and Gates (19) found the parathyroids of rabbits hypertrophied by irradiation and their blood calcium increased. Hyperparathyroidism as a clinical entity has been described by Barr, Bulger, and Dixon (20) and Wilder (21) as associated with cases of osteomalacia or osteitis fibrosa, showing negative calcium balance, bone decalcification, muscular hypotonia, and multiple cystic bone tumors. Higgins and Sheard (22) produced hyperplasia of the parathyroids of chicks grown in the absence of the ultraviolet portion of sunlight. Cod liver oil or direct irradiation prevented the hyperplasia, which was followed by rachitic bone lesions and destructive regression of the parathyroids.

The effect of parathyroid extract upon animals deprived of the antirachitic vitamin has not been recorded so far as we could learn. It seemed worth while to check the observations of Collip and Clark (9), Greenwald and Gross (7), and others concerning the effect of the extract in normal adult dogs by corresponding experiments on young vitamin D-free dogs. Our interest was aroused originally by the possible rôle of parathyroid abnormalities in the production of pyorrhea and other mouth diseases. Loosening of teeth was observed in the hyperparathyroid case of Barr, Bulger, and Dixon (20) and the defects of enamel and dentine in teeth of parathyroidectomized rats have repeatedly been described by Erdheim and his colleagues. The plan of the experiment included the study of calcium and phosphorus balances and blood content of young dogs given varying and repeated doses of potent parathyroid extract with constant diet and controlled vitamin D body content. Similar experiments upon thyroparathyroidectomized animals were planned, but are not here reported. Another short series of blood examinations was made upon several older dogs in order to test a second phase of the problem, that of the effect of metabolic reaction of the diet upon the sensitivity of the animals to parathyroid dosage. The latter phase grew out of an extended series of feeding experiments upon the relation of the acid-base equilibrium to the production and cure of bone and tooth damage in dogs.

TABLE I.
Composition of Diets.

Diet No.	Constituents.	Gm. per kg. per day.	Protein calo- ries.	Ca	P	Ca·P
			<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
12, stock diet.	Casein, commercial.	5.0	26			
	Sucrose.	4.0				
	Dry skim milk.	3.0				
	Agar.	0.4				
	Butter.	1.6				
	Lard or Crisco.	2.2				
	Canned tomato.	15.0				
4 m	Casein, commercial.	19.2	30	0.32	0.38	0.84
	Sucrose.	21.3				
	Salt Mixture 185 (McCollum).*	1.0				
	Agar.	0.4				
	Crisco or lard.	5.0				
	Cod liver oil (Squibb).	1.0				
	Dried brewers' yeast.	0.6				
11 a	Same as Diet 4 m, but with low Ca Salt Mixture 4.		30	0.27	0.69	0.39
11	Same as Diet 11a, but with Crisco or lard 2.4 gm., and butter fat 3.6, instead of Crisco or lard 5, cod liver oil 1.			0.30	0.60	0.50
18	Casein.	5.0	31	0.26-0.39	0.22	1.18-1.77
	Wheat gluten.	15.0				
	Sucrose.	21.3				
	Low P Salt Mixture 6.	1.0				
	Crisco or lard.	5.0				
	Yeast.	0.6				
	Cod liver oil (Squibb).	1.0				

TABLE I—*Concluded.*

Diet No.	Constituents.	Gm. per kg. per day.	Protein	Ca	P	Ca:P
			calo- ries. per cent	per cent	per cent	
19	Same as Diet 18, but with Crisco or lard 2.4 gm., butter fat 3.6, instead of Crisco or lard 5, cod liver oil 1.		31	0.26-0.39	0.22	1.18-1.77
20	Same as Diet 19, but with egg albumin substituted for casein.		31	0.23	0.14	1.64

* McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, **33**, 63 (1918).

EXPERIMENTAL.

Two litters of pups were used in the first attempt to note the relation of vitamin D to the characteristic effects of parathyroid extract. Both litters, designated Litters K and L, were born in the laboratory of mothers kept during pregnancy and lactation upon a standard stock diet (Diet 12, Table I) of casein, sugar, skim milk powder, agar, butter, lard, and tomato (23). At weaning, when they were 5 weeks of age, the pups were placed on an artificial diet of relatively low phosphorus but normal calcium content. This diet, No. 19, is given in detail in Table I.

The salt mixture used was based on analyses of dog milk and was made up as follows:

*Low Phosphorus Salt Mixture*⁶.

	gm.
MgSO ₄	4.5
NaCl.....	20.4
KCl.....	15.8
Ca lactate.....	133.9
Ferric citrate + 1.5 H ₂ O.....	2.0

The low calcium Salt Mixture 4, used in Diets 11 and 11 a (Table I) was a modification of the Osborne and Mendel (24) salt mixture made up as follows:

Low Calcium Salt Mixture 4.

	gm.		gm.
MgCO ₃	48.4	H ₃ PO ₄	117.0
Na ₂ CO ₃	68.4	HCl.....	53.4
K ₂ CO ₃	241.0	H ₂ SO ₄	9.2
KI.....	0.020	Citric acid + H ₂ O....	53.0
MnSO ₄	0.079	Fe citrate + 1.5 H ₂ O..	6.34
NaF.....	0.248	K ₂ Al ₂ (SO ₄) ₄	0.024

The growth of the dogs was satisfactory, although only two of each litter were given any source of vitamin D. The other animals in spite of a fair allowance of butter fat, early developed clinical signs of rickets or a similar bone defect. Radiographs of wrists and ankles and blood calcium and phosphorus determinations were made regularly during the 2nd and 3rd months of life. Early in the 4th month the parathyroid injections were begun in four of Litter L. At this time all of the eight dogs of the litter, except Dogs 115 and 116, were showing enlarged epiphyses, somewhat lowered serum calcium and inorganic phosphate, and the knobby joints and bowed legs of active rickets. Dogs 115 and 116 were given 1 mg. of irradiated ergosterol in 1 ml. of corn oil daily, in addition to the basal diet. The ergosterol was supplied to us in crystalline form, already irradiated, by the laboratory of E. R. Squibb and Sons. The crystals were at once dissolved in neutral corn oil, 1 mg. of ergosterol per ml. of oil. The Squibb Biological Laboratories had tested the material and found it potent with rats, using the line test in amounts of 0.0001 mg. daily for 5 days. This test was repeated in this laboratory 2 months later, with the corn oil solution of the irradiated material, with the same technique. It was found that a good line test could be obtained by using 0.25 mg. of our oil dilution of the ergosterol daily for 5 days on standard rachitic rats. This corresponds to 0.00025 mg. of the original crystals. We did not make the test with smaller doses, which may of course have been effective. Our oil dilution therefore proved to contain just 100 times the antirachitic value of Squibb's cod liver oil with which it was compared in our tests upon both dogs and rats. We thus happened to use viosterol of the recognized strength (25).

The dosage given Dogs 116 and 115 was thought to be far in excess of that necessary, since 1 gm. of cod liver oil daily per kilo had been shown in this laboratory previously to be sufficient to

protect young dogs from bone abnormalities. In the later series shown in Table III, this quantity of the ergosterol nevertheless proved inadequate. The latter animals were fed a diet (No. 20) of lower phosphorus content than that given Litters K and L, and it may be that under such severely restricted intake of that element, normal ossification cannot be promoted in the dog by any amount of vitamin D.

Dogs 117 and 119 were given 0.45 gm. of ammonium chloride per kilo per day so as to produce an acid urine, pH 5.6 to 6.2, and Dogs 120 and 121, 0.5 gm. of sodium carbonate per kilo per day to produce an alkaline urine, pH 8.0 or more. On April 16, injections of 10 units of parathyroid extract, Squibb, daily were given to Dogs 116, 118, 119, and 120 for 10 days, then 20 units daily for 3 days, none for 2 days, 20 units again for 11 days, then 30 units for 8 days, and on the last day before the animals were sacrificed, 50 units. Thus the extract was administered on 33 of the last 37 days. However, Dog 116 was so obviously suffering almost at once from overdosage that the dosage was kept lower and given on only 22 days.

Effect of Vitamin D with Small Repeated Doses of Parathyroid Extract.

As may be noted in Table II, the response of Dog 116, due probably to the vitamin D being fed, was excessive and finally fatal. The increase in serum calcium was as high as 9.8 mg. per cent on the last day and was never lower than 3.1 when 20 units or more were given. Serum calcium was determined by the Clark-Collip (26) modification of the Kramer-Tisdall method, and inorganic phosphate by that of Fiske and Subbarow (27). The corresponding vitamin D-free Dog 118, with urine neutral in reaction, remained at a very slightly increased serum calcium level and showed a maximum increase of 2.4 mg. per cent. Dog 116 lost appetite almost at once, although no vomiting or lethargy was observed until the terminal day.

Dog 119, urine acid in reaction, and vitamin D-free, responded even less to the parathyroid hormone than did Dog 118, but Dog 120, urine alkaline in reaction, and vitamin D-free, died of overdosage after only ten doses of 10 units daily and three doses of 20 units daily. No high serum calcium values were observed but

TABLE II.

Effect of Parathyroid Extract Injection upon You Dog with and without Antirachitic Vitamin D (Diets 18 and 19)
 Figures for Ca calcium and inorganic phosphor expressed in mg. per 100 cc. of blood.

Dog No.	Vitamin D intake.	Parathyroid extract dosage.	Date.	Hrs. after last injection.	Blood.				
					Serum Ca.	Increase in serum Ca.	Inorganic P.	Change in inorganic P.	Hemo-globin. per cent
1116 ♀, 4 kilos, age 3.5 mos., Litter L.	1 gm. cod liver oil per kilo Mar. 26-Apr. 5. 1 mg. irradiated ergosterol daily Apr. 6-22. 1 mg. irradiated ergosterol every other day Apr. 23-May 13.	None.	1939 Apr. 11		12.0		4.7		98
		10	" 16	4	12.6	0.6			92
				8	12.1	0.1	5.2	+0.5	93
				12	12.8	0.8			
		10 daily Apr. 16-26.	" 22	8			5.4	+0.7	87
		20 " " 27-29.	" 29	8	15.1	3.1	5.2	+0.5	105
		None Apr. 30-May 1.							
		20 daily May 2-4.							
		None May 5-9	May 7		10.5		2.5		114
			" 8				3.3		95
1 mg. irradiated ergosterol daily May 14-22.	20 daily May 10-16.	" 14	8	15.2	4.7	3.9	+1.0	111	
	None May 17-19.								
	20 daily May 20-21.	" 21	8-10	14.3	3.8	3.7	+0.8	95	
	30 May 22.								
	50 " 23.	" 23	15	20.3	9.8	4.3	+1.4		
		Dying of overdosage.							

TABLE II—*Concluded.*

Dog No.	Vitamin D intake.	Parathyroid extract dosage.	Date.	Hrs after last injection	Blood.				
					Serum Ca	Increase in serum Ca.	Inorganic P.	Change in inorganic P.	Hemo-globin per cent
120 ♀, 6 kilos, Litter L.	No vitamin D. 0.5 gm. Na ₂ CO ₃ per kilo per day added to make diet alkaline.	<i>units</i> None 10 Apr. 16. 10 daily Apr. 17-26. 20 " " 27-29. None Apr. 30-May 2. Died of overdose May 2.	1929 Apr. 11	4 8 12 8 1 8 At death.	10 92	0 30	5.16		95
			" 16		11 22	0 58	4 60		90
			"		11 50	0 70		-0 56	109
			" 22		11 62	0 37	4 32	-0 84	87
			" 29		11 29	1 18	3 86	-1 30	96
			"		12 10	0 72	4 0	-1 16	95
			May 1		11 64	-1 10	1 55	-0 61	95
			" 2		9 82	-1 71	6 47	+1 31	92
					9 21				
					9 30		3 91		95
121 ♂, 5 kilos, Litter L.	No vitamin D. Na ₂ CO ₃ as for Dog 120.	None. Died of pneumonia May 2.	Apr. 11		11 57		3 21		97
			" 25		11 66			93	
			May 2						
114 ♀, 4 kilos, Litter L.	No vitamin D.	None.	Mar. 7				4.55		105
			Apr. 11	12 04		4 29		99	
			" 25	10 35		4 91		100	
			May 2	10 87		3 55		92	
			" 9	10 65		3 44		105	
			" 14	11 43		3 86		107	
			" 21	10 81		3 55		87	

115 ♂, 4-6 kilos, Litter L.	1 mg. irradiated ergosterol daily Mar. 7-May 21.	None.	Apr. 11 " 25 May 2 " 9 " 14 " 21		11.59 11.04 10.39 11.03 11.61 11.48		5.63 5.12 6.10 5.53 4.67 4.63	91 90 88 100 110 83
109 ♀, 5 kilos, age 5.5 mos., Litter K.	1 gm. cod liver oil daily per kilo Mar. 23-May 22. 2 mg. irradiated ergosterol daily, instead of cod liver oil, May 23-29.	None. " 60 May 27. 100 May 28. Died of overdosage May 29.	May 3 " 17 " 27 " 28 " 29		10.43 10.24 11.48 15.58 15.14 14.75		3.24 3.92 3.26 3.70 7.04 9.79	93 99 114 114
111 ♂, 11 kilos, Litter K.	No vitamin D.	None. 30 daily May 15-17. None May 18-26. 60 May 27. 100 May 28. Good condition.	May 3 " 17 " 27 " 28 " 29	8 14 14 19	10.43 10.64 11.83 11.43 12.63 12.36	0.21 -0.4 0.8 0.53	3.94 4.10 3.94 3.75 4.34 5.57	113 99 125 105 102
112 ♀, 7.5 kilos, Litter K.	1 gm. cod liver oil daily per kilo, Mar. 23 on.	None. 30 daily May 15-17. Died of overdosage May 17.	May 3 " 17	8	12.36 8.83	-3.53	5.10 10.6	106 141
113 ♂, 8-9 kilos, Litter K.	No vitamin D.	None. " 60 100 Good condition.	May 3 " 17 " 27 " 28 " 29	14 14 20	10.91 11.17 11.74 14.26 13.82 12.98	2.52 2.08 1.24	3.10 2.37 2.16 2.66 4.92 5.61	113 111 102 91 90

only the lowered terminal figures. Vomiting, bloody diarrhea, and extreme relaxation and coma were the symptoms noted in the last 3 days of this dog's life. This exception to the apparent immunity enjoyed by the other vitamin D-free dogs raised the suspicion that the alkaline reaction of the diet conducted to increased susceptibility. Accordingly, another series of experiments was tried upon adult dogs to test this point alone. The results, shown in Table IV, and later discussed, bore out the conclusion that the alkaline reaction increases the response to parathyroid extract.

The four remaining dogs of Litter L, Dogs 114, 115, 117, and 121, were maintained as controls and calcium and phosphorus balances as well as blood analyses carried out on them regularly. As might be expected, Dog 115, which received 1 mg. of irradiated ergosterol daily, showed higher blood inorganic phosphate figures than the other three, and also greater retention of both calcium and phosphorus, data as to which will be reported later. Dog 121 died of pneumonia before the end of the study.

Results with Large Single Doses of Parathyroid Extract.

Litter K, four pups, was placed upon the same diet as Litter L at weaning, and at approximately 3 months of age all the dogs showed severe rickets, by radiograph and blood composition. Dogs 109 and 112 were then given 1 gm. of cod liver oil (Squibb) per kilo daily, and Dogs 111 and 113 left without treatment. As shown in Table II, 2 months later Dogs 111 and 112 were each given three doses of 30 units daily. Dog 112 died with all symptoms of overdosage on the 3rd day. Again, as with Dog 120, only the final low calcium-high phosphate stage of the blood picture was detected. Dog 111 remained in good condition and showed practically no rise in blood calcium.

Later, Dog 109 was given 2 mg. of irradiated ergosterol daily instead of the cod liver oil, and all three remaining animals, Dogs 109, 111, and 113, were given 60 units of parathyroid extract, followed 24 hours later by 100 units. Dog 109 died of overdosage on the 2nd day, with continuous high blood calcium. Dog 111 showed very little increase in blood calcium and no symptoms of overdosage. Dog 113 showed no symptoms of overdosage, but a moderate increase in blood calcium. This

increase is the chief exception we have found to the lack of response of vitamin D-free dogs to the extract. The animals in Litter K were older than any of the other pups used for this work, and it is possible that Dog 113 had obtained more light treatment than the others. All of the dogs had had an attack of distemper 2 or 3 months earlier, and Dog 113 had been the first to become sick and had been given several sun baths during the illness. This was not done with the others to the same extent. This may account for the response of Dog 113 to the parathyroid extract, although the blood phosphorus remained low until after the second dose of extract.

Second Test of Effect of Viosterol on Parathyroid Response.

Another group of young pups was tried later with similar results, which are shown in Table III.¹ Diet 20 was used instead of Diet 19, in order to produce a more excessive Ca:P ratio, and the injections first made at 10 weeks of age. Dogs 140, 142, 143, 144, 146, and 147 were of Litter P which was born and reared in the laboratory under the same circumstances described for Litters K and L. Dogs 134 and 135 were of a similar litter, Litter O, and Dog 148 was a stray pup of unknown previous history, but apparently the same age as the others. Antirachitic vitamin was given only to Dogs 140, 142, 143, 147, and 148. These pups were given 1 mg. of irradiated ergosterol daily, a dose which proved later to be insufficient to prevent rickets on this low phosphorus diet. Dogs 147 and 148, even after several weeks of 5 mg. of viosterol daily, showed rachitic paralysis and greatly enlarged wrist joints. On autopsy severe rickets, or osteitis fibrosa, was evident by macroscopic inspection. Microscopic studies of these cases are still under way. Five of the dogs, Nos. 140, 142, 143, 144, and 146, were given 30 units of parathyroid extract daily beginning June 28. Dogs 140, 142, and 143 died on July 2 and 3, after four injections, all with symptoms of overdosage. These were the three vitamin D-fed dogs. Dog 144, vitamin D-free, received eight doses of 30 units each without showing ill effects. Dog 146, also vitamin D-free, died after seven doses of 30 units each.

¹ The earlier portion of the analytical work on this group of dogs was done by Ethel Curry.

TABLE III.
Effect of Parathyroid Extract upon Dogs, 10 Weeks of Age and Later, with and without Antirachitic Vitamin D (Diet 20)
 Figures for serum calcium and inorganic phosphorus are expressed in mg. per 100 cc. of blood.

Dog No.	Vitamin D intake	Parathyroid extract dosage.	Date.	Blood.			Remarks.
				Serum Ca.	Change in serum Ca.	Inorganic P.	
142 ♂, 2.8 kilos.	1 mg viosterol daily.	None. 30 daily, June 28-July 1.	1929 June 11	11 60		5 1	Died of overdose on July 2, after 4 doses of 30 units.
140 ♀, 2.8 kilos.	Same.	None 30 daily, June 28-July 1.	" 11	11 74		4.9	" "
143 ♂, 3.1 kilos.	Same.	30 daily, June 28-July 1.	July 3	13 39	+1.8	3.3	Died of overdose on July 3, after 4 doses of 30 units.
144 ♂, 3.0 kilos.	No vitamin D.	Before para- thyroid injec- tion. 30 daily, June 28-July 5.	June 27	11 53		1.8	
		None. "	July 5	10 15	-1.38	3 0	Severe rickets. No ill effect of parathyroid injection.
		"	Sept. 28	10 00			
		"	Oct. 14	7.75			
		30	" 21	8 35			
			" 22	8.92	+0 57		
5.8 kilos		60	" 24	11.20	+2 85		No symptoms of over- dosage.
		None	" 28	8 64			

147 ♂, 3.2 kilos.	1 mg. viosterol daily June 30-Aug. 20. 5 mg. daily Aug 21- Oct. 20.	None. " " " "	June 19 July 5 Sept. 28 Oct. 14 " 21 " 22	10 23 12 04 12 59 10 28 9 90 13 35	+4.45 +6.27	5.4 4 3	
5.2 kilos.		30 30 None.	" 24 " 28	16.17 9 97			Loss of appetite, lethargy.
148 ♀, 3.2 kilos. 5.1 kilos.	1 mg. viosterol daily June 30-Aug. 20. 5 mg. daily Aug 21- Oct. 5.	" " "	July 5 Sept. 24	10 88 11 85		1.7 4 4	Severe rickets. Rachitic paralysis.
134 ♂, 3.0 kilos. 2.5 kilos.	No vitamin D.	" " "	June 27 July 5 Sept. 24	10 97 10 83 11 66		2 3 4.4	Severe rickets. Healed by voluntary fasting.
135 ♂, 3.0 kilos. 3.0 kilos.	No vitamin D.	" " "	June 27 July 5 Sept. 24	11 35 11 44 11 05		2.8 4 6	Severe rickets. " Healed by voluntary fasting.
146 ♀, 2.1 kilos.	No vitamin D.	Before thyroid in- jection.	June 27	9.58			Died on July 6 after 7 doses of 30 units each.

This was the smallest dog in the lot. Regular blood analyses were not obtained, but as may be seen in Table III, Dog 143, with vitamin D, showed an increase in serum calcium on parathyroid injection, and Dog 144, vitamin D-free, did not. Calcium and phosphorus balances were made on all these young dogs before and after parathyroid injection, but these data are not presented at this time.

Evidently the younger animals are more sensitive to the action of the hormone, and particularly to that of the combined vitamin and hormone. A striking similarity is evident between the symptoms of parathyroid overdosage here observed and those described as caused by overdosage with viosterol, the "hypervitaminosis" of Hess, Lewis, and Rivkin (12), of Harris and Moore (28), and others.

Several months later two of these dogs were again given the extract, 30 units followed 48 hours later by 60 units, in the case of vitamin D-free Dog 144, and 30 units followed at the same interval by 30 units to vitamin D-rich Dog 147. As may be seen in Table III, the serum calcium rise in the latter dog was 3 to 6 times as great as in the former, even on larger dosage. Both of these animals on radiographic examination gave evidence of healed severe rickets. Thus the indifference to parathyroid extract would seem to be independent of the presence of active bone disease, but dependent upon the absence of a store of vitamin D.

The behavior of this group of dogs indicates that the earlier observations were not conditioned by the sex of the animals used, since these males proved to be just as susceptible to parathyroid overdosage as did the females, Dogs 116, 109, and 112, in the earlier experiments.

Calcium and phosphorus balances upon all these dogs, eighteen animals in all, have been made, both with and without concurrent administration of parathyroid extract. The results of these studies will be published later.

Effect of Reaction of Diet.

As noted previously, Dog 120, a young vitamin D-free animal, succumbed to parathyroid overdosage after injections of 160 units distributed over 13 days. Two others of the same litter, Dogs 118 and 119, showed little effect from a total of 670 units in

37 days. Since Dog 120 was given sodium carbonate to make the urine alkaline, while that of Dog 118 was kept neutral and that of Dog 119 made acid with ammonium chloride, it was thought possible that the sodium carbonate exerted an injurious effect in conjunction with the parathyroid treatment. Accordingly a few experiments were made to test this theory.

As shown in Table IV, Dog 15, an old male which had been in the laboratory nearly 2 years, subsisting upon the standard stock diet, Diet 12, and without access to sunlight, responded by somewhat less than the expected increase in serum calcium to injections of the parathyroid extract. This animal's blood phosphate was unusually low at the same time. In Period 2, after being given 5 mg. of irradiated ergosterol daily for 5 days, the level of serum calcium was found to be raised but the response to parathyroid was even less than before. In Period 3 sodium carbonate was added to the diet in sufficient amount to produce a urinary pH of 8.0 or more. The rise in serum calcium following injection of the parathyroid extract on this diet was remarkably increased, and the dog showed symptoms of overdosage. Indeed for several weeks, this animal ate very little and lost weight. In Period 4 ammonium chloride was added to the diet so that the urinary pH was close to 5.8. The response to the parathyroid hormone was less than in the alkaline period but greater than in the neutral periods. In this series, the extract was injected at the same hour each evening and the blood samples taken 13 hours later the next morning. This animal, tested again several months later on a neutral diet, responded with calcium increases almost the same as those seen in Period 1.

Dogs 1 and 22, adult females kept under the same conditions as Dog 15, showed varying response to the hormone on neutral diet, and Dog 1, about the same on acid diet as in Period 2 on neutral diet.

Dogs 94 and 95, young animals reared on artificial high phosphorus diets, Nos. 11 and 11 a, differed both as to reaction and vitamin D content. Only small doses of extract were given and the order of response was similar to that found in Litters K and L on low phosphorus diet.

Dog 79, reared in the laboratory on an adequate artificial diet, No. 4 m, made alkaline by sodium carbonate, showed a rather

TABLE IV.
Effect of Metabolic Reaction of Diet upon Response of Dogs to Parathyroid Extract.
 Figures for serum calcium and inorganic phosphorus are expressed in mg. per 100 cc. of blood.

Dog No.	Diet.	Date.	Parathy- roid extract dosage.	Blood.			
				Serum Ca.	Increase in serum Ca	Inorganic P.	Change in inorganic P
15 ♂, 11 kilos, adult.	Period 1. Diet 12. Adequate stock diet with no addition of vitamin D. Period 2. 5 mg. irradiated ergo- sterol daily May 24-28.	1929	units				
		May 21	None.	11 03		2 08	
		" 22	100	14.56	3 53	2 03	-0.05
		" 23	100	15 54	4 51	1 92	-0.16
		" 27	None.	12 45		1 93	
	Period 3. Same as Period 1 but made alkaline by addition of 0.5 gm. Na ₂ CO ₃ per kilo per day.	" 28	100	14.48	2 03	1 90	-0.03
		" 29	100	14 03	1 58	2 35	+0.42
		June 12	None.	10 60			
		" 14	100	16 07	5.47	2 43	
		" 15	100	21 19	10 59	3.46	+1 03
	Period 4. Same as Period 3 but 0.45 gm. NH ₄ Cl per kilo per day substituted for Na ₂ CO ₃ . Period 5. Same as Period 1.	" 24	None.	11 25		1 61	
		" 27	100	15 62	4 37	2 30	-+0.69
		Oct. 8	None.	11 40		3 12	Inorganic P de- termined on serum instead of whole blood in Period 5.
		Oct 10	100	14 90	3 50		
		" 14	None.	10 36		3.80	
	" 17	100	14.20	3 84	4.25	+0 45	

1 ♀, 9 kilos, adult.	Period 1. Same as Dog 15, Period 1.	Apr. 29 " 29 " 30 May 1*	None. 20 40 30	11 52		1 31	
	Period 2. Same as Dog 15, Period 2.	" 21 " 22 " 23 " 27 " 28 " 29	None. 100 100 None. 100 100	11 75 10 06 14 47 15 10 11 48 14 48 13 15	0 23 4 41 5 04 3 00 1 01	1 27 2 24 2 36 2 10 1 08 2 41 2 43	-0 04 +0 12 -0 14 +1 33 +1 35
	Period 3. Same as Period 1 but NH ₄ Cl added as in Period 4, Dog 15.	June 13 " 14 " 15	None. 100 100	10 15 12 45 14 13	2 30 3 98	2 98 2 57 3 88	-0 41 +0 90
22 ♀, 9 kilos, adult.	Period 1. Same as for Dog 15, neutral reaction.	May 21 " 21 " 22 " 27 " 27	None. 100 100 None. 100	11 8 12 7 15 98 11 39 11 92	0 9 4 18 0 53 0 79 2 38 3 53	2 54 2 54 2 27 2 13 3 02 2 66 2 45 2 83	-0 27 +0 89 +0 53 +0 32 +0 70
	Period 2. Same as Period 1.	" 28 June 14 " 15	100 100 100	12 18 13 77 14 92			
	Period 3. Same as Period 1.						
94 ♂, † 7 kilos, age 7 mos.	Diet 11, no vitamin D, acid reaction (0.46 gm. NH ₄ Cl per kilo per day).	Apr. 22 " 26 " 30	None. 20 30	11 58 10 35 12 35	-1 23 0 77	3 54 3 62 3 47	+0 08 -0 07

TABLE IV—Concluded.

Dog No.	Diet.	Date	Parathyroid extract dosage.	Blood.			Change in inorganic P.
				Serum Ca.	Increase in serum Ca.	Inorganic P.	
95 ♂, † 8 kilos, age 7 mos.	Diet 11 a, containing cod liver oil, 1 gm. per kilo per day, neutral.	1929 Apr. 22	units None.	12.54		5.27	
		" 26	20	13.07	0.53	5.82	+0.55
		" 30	30	12.80	0.26	4.91	-0.36
79 ♀, 9 kilos, age 11-13 mos.	Diet 4 m, containing 1 gm. cod liver oil per kilo per day. 0.5 gm. Na ₂ CO ₃ per kilo per day since weaning.	Apr. 22	None.	11.83		3.17	
		June 24	"	10.32		2.68	
		" 27	100	15.90	5.58	3.90	+1.22

* Blood sample taken 4 hours after parathyroid extract injection. All others in this table, except as noted, taken 13 hours after injection.

† Blood samples taken from Dogs 94 and 95 6 hours after parathyroid extract injection.

large serum calcium rise as a result of the one parathyroid injection given. This dog had had the alkali from weaning.

An average of all changes in serum calcium and inorganic phosphate is shown in Table V both for the vitamin D influence and that of the metabolic reaction of the diet. It is plain that vitamin D and alkaline reaction of diet, together or separately, cause considerably larger increases in both calcium and inorganic phosphate phosphorus than any of the other conditions. The viosterol appears to increase blood phosphate more noticeably than does the alkali, although this may possibly be due to the fact that younger dogs were used for the former test. The well known fact that ammonium chloride alleviates tetania parathyreopriva might incline one to expect this substance or any other acid agent to exaggerate the toxic symptoms caused by the parathyroid extract, and to assume that a fixed base such as sodium carbonate would decrease the severity of such symptoms. That the administration of ammonium chloride produces an acidosis is generally believed, and as a result, *ionic* calcium may be increased. But *total* serum calcium is not affected by such a procedure, as may be seen in the data concerning Dogs 117 and 119 in Table II and Dogs 94, 1, and 15 in Table IV.

The contradictory results which have been reported as to diffusible calcium in the blood of parathyroidectomized animals (10, 11) offer no assistance in the interpretation of the experiments here reported. The reports (29) indicating that alkalosis does not exist at any time following parathyroidectomy in dogs make it difficult to correlate the toxic effect of sodium carbonate and parathyroid extract since certainly no acidosis has been assumed to exist in the tetanic state. If alkalosis is among the overdosage phenomena of parathyroid extract, its existence has not hitherto been noted. There is now under way in this laboratory a series of experiments designed to test this hypothesis. The possibility of a specific effect of the sodium ion must also be considered, since upset of the equilibrium among the four cations common to body fluids has often been suspected as contributory to production of tetany or its opposite.

The interpretation of the findings concerning the intensifying effect of vitamin D upon the toxic action of parathyroid extract in the light of current theories of parathyroid function is not obvious.

The most plausible theory is that of Greenwald (5) who believes that the parathyroid glands furnish some substance to the blood

TABLE V.

Summary of Changes in Serum Calcium and Inorganic Phosphate, Due to Parathyroid Extract, in Dogs with and without Vitamin D and on Diets of Varying Reactions.

Dog No.	No. of experiments for group.	Body weight.	Age.	Diet.	Parathyroid extract dosage.	Average increase in serum Ca.	Average change in inorganic P of whole blood.
		kg.	mos.		units	mg. per 100 cc.	mg. per 100 cc.
118 119 120 111 144	30	4-11	3-6	Low phosphorus, no vitamin D.	10-50	0.59	-0.28
111 113 144	7	8-11	6	Low phosphorus, no vitamin D.	60 or 100	1.64	+1.02
112 116 147	11	4-7 5	4-6	Low phosphorus, with cod liver oil or irradiated ergosterol.	10-50	3.65	+3.18
109	3	5	6	" "	60 or 100	3.09	+2.79
15 1 22	14	9-11	Adult.	Adequate diet, neutral reaction.	100	2.75	+0.41
15 79	3	9-11	Adult.	Adequate diet, alkaline reaction.	100	6.99	+0.98
15 1	3	9-11	Adult.	Adequate diet, acid reaction.	100	3.54	+0.35

which is, or is necessary for the production of, that which renders calcium soluble in the blood serum. Further it has been postulated by Greenwald and Gross (4), by Brougher (2), by Jones (1), and by

Hess, Lewis, and Rivkin (12) that vitamin D stimulates the production of this substance by the glands. But the results here reported indicate apparently that the stimulation is not to the production of secretion but to the intensity of effect of that secretion. The blood calcium and nervous excitability of the vitamin D-rich Dogs 116, 112, and 109 were no greater than those of their vitamin D-free litter mates, Dogs 118, 119, 120, 111, and 113 before the parathyroid injections were begun. This might be interpreted to mean that their parathyroid glands were about equally active. But when the parathyroid extract was given, the recognized results of the parathyroid hormone were intensified in those dogs having vitamin D. This effect must have taken place at the site of the activity promoted by the secretion and can scarcely be looked upon as directly related to the condition of the animal's parathyroid glands. However, since damage to these glands has been noted in vitamin D-free animals (22, 30), the hypothesis of inactivity of the vitamin D-free dogs' parathyroids cannot be ignored. Experiments involving histological study of the glands of such dogs are now under way in the hope of learning the facts on this point.

Again, the primary effect may be in the intestine where the permeability to both calcium and phosphorus may be changed by the hormone in one direction and by the vitamin in the other. If the vitamin decreases the ease with which one or both of these elements is excreted into the intestine, the calcium content of the blood might rise more strikingly as a result of the influence of the parathyroid extract in the dogs given vitamin D than in the vitamin D-free animals in which such excretion is abnormally easy.

In any case, it appears to be of clinical significance that vitamin D and alkali increase the response to parathyroid treatment. In recent reports of hyperparathyroidism, treatment by cod liver oil or viosterol and calcium salts has been suggested. Such treatment should be inaugurated cautiously, and possibly only after operation upon the glands if tumor of any type is suspected. The treatment of tetany of any origin, of edema, or any of the other conditions for which the extract has been used should be carried on with these dangers of overdosage in certain cases constantly in mind.

SUMMARY.

1. Young dogs reared on artificial diets with a Ca:P ratio 1.18 to 1.64, without vitamin D, have been found to respond but little to the injection of a potent parathyroid extract. Serum calcium of such animals is only slightly raised by the extract, and clinical symptoms of overdosage are lacking. This lack of response is independent of the presence of active rickets or other bone disease.

2. Similar dogs, given cod liver oil or viosterol, upon parathyroid extract injection show abnormally large increases in serum calcium and inorganic phosphate and the rapidly fatal symptoms of overdosage. This may occur even when the animal shows active clinical rickets, produced by low phosphorus intake in spite of viosterol dosage.

3. Both young and adult dogs given mixed and artificial diets of normal Ca:P ratio, low phosphorus and low calcium contents, and either with or without vitamin D respond with abnormally high serum calcium and toxic symptoms to parathyroid extract dosage, if sufficient sodium carbonate (0.5 gm. per kilo per day) is given by mouth to make the urine alkaline. Similar treatment with ammonium chloride only slightly affects the response to the hormone.

4. The bearing of these findings upon the theories of parathyroid and vitamin D functions is discussed, and certain clinical aspects of the experiments pointed out.

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THE SPONTANEOUS OXIDATION OF DIALURIC ACID.

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INTRODUCTION.

It is very desirable that other autooxidative processes be studied with the same degree of thoroughness as that of cysteine by Mathews and Walker, Warburg, Michaelis, and others, and that of glutathione by Hopkins and his students. These oxidations imitate so closely many features of cell respiration, particularly in their catalysis by iron and some other metals and their susceptibility to poisoning by cyanides, as to suggest that they take part in the respiration of living matter. Since the nucleus plays an important rôle in respiration—as is shown by the fact that such enucleated cells as the red blood corpuscles of mammals have almost no respiration, while the same nucleated cells have a normal respiration, and also by the studies of Mathews (1), Loeb (2), Lillie (3), Osterhout (4), and many others on starfish eggs, leucocytes, plant, and other cells, studies which have shown that some sort of an oxidase appears to originate in the nucleus—it seemed desirable to study the autooxidation of some nuclear constituent. Among such constituents nucleic acid naturally occurs to one. But this acid oxidizes itself in the air only very slowly if at all. It contains, however, several groups in its molecule which by themselves are readily oxidized in the air after they have undergone a preliminary oxidation. One of these would probably be the unstable hexose of an unknown kind in nucleic acid but it has not yet been possible to secure this for experimentation, as it decomposes so readily. The purines and pyrimidines, however, can be obtained from nucleic acid and among their decomposition products there are some which oxidize in the air very readily.

Some 9 years ago Professor Mathews, in conjunction with his

student, Mr. George Hoeck, started the investigation of the spontaneous oxidation of dialuric acid, an oxidation product of uric acid. After a few months of work, the illness of Mr. Hoeck suspended the study before it was ready for publication. At Professor Mathews' suggestion, and as Mr. Hoeck's continued illness has made it impossible for him to go on, I rebegan the investigation.

The work has been made possible by the generosity of Professor John Uri Lloyd, who has for many years been interested in the rôle of iron in cell respiration and who thought that further work along these lines would be valuable. I am deeply indebted to Professor Lloyd for his generosity in making it possible for me to undertake the study under the guidance of Professor Mathews. The chemical aspects of the problem of the catalytic action of iron have been under the direction of Professor H. S. Fry; and one study on the oxidation of citric acid under the influence of iron and ultra-violet light, begun at the suggestion of Professor Lloyd, has already been published by Fry and Gerwe (5).

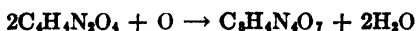
When the work of this investigation was being completed, Richardson and Cannan (6) published the results of some investigations which they had made on the equilibrium existing between dialuric acid and alloxan in aqueous solution. They determined the equilibrium potentials of the reversible oxidation-reduction system, dialuric acid-alloxan, for a pH range from 1 to 6. They also made a few observations upon the rates of autooxidation of dialuric acid in buffered solutions and found that between pH values of 5 and 2 the rates were very great but fell away below this. No observations were published upon the rate of oxidation for any values above pH 6.

The power of dialuric acid as a reducing agent has been employed by Ruhemann (7) in the preparation of hydrindantin from triketohydrindene hydrate (ninhydrin), the reaction involving the reduction of the ninhydrin to hydrindantin by the dialuric acid and its oxidation to alloxan. Harding and Warneford (8) have repeated this observation and have employed dialuric acid in a study of the action of reducing agents on the ninhydrin reaction with ammonium salts. They found that dialuric acid was the most efficient of organic reducing agents in reducing ninhydrin to hydrindantin, which, on warming with an ammonium salt, gave a

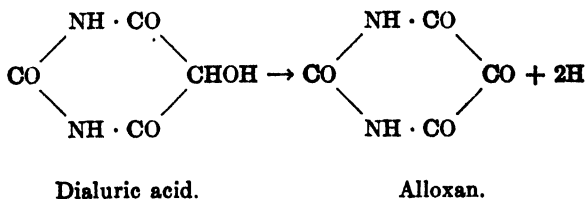
compound having a blue color identical with that formed by an α -amino acid and ninhydrin.

The oxidation of dialuric acid is also of interest in a consideration of the metabolism of uric acid. By the hydrolysis and oxidation of uric acid, dialuric acid and alloxan are formed. This destruction of uric acid is brought to pass chiefly in the liver or kidneys. Ascoli and Izar (9) showed that in the presence of carbon dioxide the liver reforms uric acid out of dialuric acid and urea. It would seem probable from these observations that the liver decomposed uric acid to dialuric acid and urea, and resynthesized them, under certain conditions, due possibly to a change in reaction, for which the carbon dioxide might be responsible. It was the object of this part of the investigation to discover the effect of variations in reaction on the oxidation of dialuric acid.

It is known that dialuric acid oxidizes spontaneously. It becomes red in color in the air, absorbs oxygen, and passes into alloxantin.



In acid solution, dialuric acid and alloxan may readily be converted into one another. The change is one involving 2 equivalents of hydrogen.



The addition of 1 equivalent of an oxidizing agent to dialuric acid, or of 1 equivalent of a reducing agent to alloxan, leads to the separation of the less soluble substance, alloxantin, a compound of a molecule of dialuric acid with a molecule of alloxan and which is believed to be extensively dissociated into its two components in aqueous solution. Alloxantin is also formed when solutions of these two reactants are mixed in equivalent proportions.

EXPERIMENTAL.

A. Preparation and Properties of Dialuric Acid.

The method of making dialuric acid was that of Biltz and Damm (10) with certain modifications. A mixture of 30 gm. of uric acid, 60 gm. of concentrated hydrochloric acid, and 60 cc. of water is oxidized by the slow addition of 8 gm. of finely pulverized potassium chlorate with shaking at 30–40°. This should take about 45 minutes. For the removal of free chlorine air is passed through the solution for 30 minutes. Then 50 cc. of concentrated hydrochloric acid and 50 gm. of crystallized stannous chloride are added and the deep yellow solution boiled. It is now cooled, finally with ice water, and stirred vigorously, so that deposition sets in, and the solution goes over into a thick, pasty mass. It is recommended to inoculate with some solid dialuric acid, if such be on hand. The crop of dialuric acid crystals sinks in a somewhat remarkable manner, if the substance is allowed to separate slowly from the undisturbed liquid. After a few hours it is filtered with suction, washed with water containing hydrochloric acid, with water, alcohol, and finally ether, and dried in a vacuum desiccator. This product will contain some tin. One can free it from that by taking it into solution with boiling water, weakly acidified with hydrochloric acid, saturating with hydrogen sulfide (warm temperature), and crystallizing the filtrate.

The writer found it advantageous to prepare the potassium salt of the acid as this is more stable than the acid itself and can be preserved, and the acid prepared just when needed. The method employed was a modification of that just described. After the oxidation of the mixture of uric acid, hydrochloric acid, and water has been completed and the chlorine expelled, 50 cc. of concentrated hydrochloric acid and 12 gm. of granulated zinc are added. When the zinc has dissolved, the solution is filtered, neutralized with a saturated solution of potassium hydroxide, and more potassium hydroxide added to complete precipitation of the potassium dialurate. The precipitate is filtered with suction, washed with water, and dried. To prepare dialuric acid, the potassium dialurate is dissolved in concentrated hydrochloric acid and water and crystallized. The crystalline product, containing some potassium chloride is then washed with water containing hydrochloric acid,

water, alcohol, and ether as before and dried in a vacuum desiccator. For further purity the dialuric acid can be recrystallized from water acidified with hydrochloric acid.

Dialuric acid crystallizes in colorless, mostly short, firm prisms, or by quicker crystallization in short, thin leaflets, whose ends are frequently rounded. The substance reddens on heating in a melting point tube to 180° and melts at $214\text{--}215^{\circ}$ with frothing.

Dialuric acid shows a very acid reaction, and forms salts with 1 equivalent of the metals, which are split neither by carbonic nor acetic acids. Completely dried preparations remain for a long time unchanged in dry air. Damp preparations are unstable and oxidize with the loss of heat. If the preparations be pure they will not redden; they will, however, if they contain alkali metal salts. The damp alkali metal salts of dialuric acid and especially the methylated dialuric acids decompose in air.

B. Influence of the Reaction on Speed of Oxidation.

1. *Apparatus.*—The blood gas apparatus of Barcroft and Roberts (11) was used for the determination of the amount of oxygen absorbed by the oxidation of the dialuric acid. In this apparatus the volume of gas absorbed is calculated from the pressure change caused in a differential manometer. The manometer is a U-tube, the tubing of which is 1 mm. bore, provided with stop-cocks through which communication may be made with the outside air, and connected with two small pear-shaped bottles, in either of which the reactive substance may be placed. The manometer is filled with clove oil of specific gravity 1.032, chosen so that 10,000 mm. of the oil equal 760 mm. of mercury.

The apparatus was first standardized by the directions given by Barcroft and Higgins (12), the following constants being determined: (1) The sectional area, A , of the two manometer tubes was found to be 1.524 sq. mm. (2) The capacity, V , of each bottle was found to be 32.21 cc. for Bottle I, and 32.40 cc. for Bottle II. When oxygen is absorbed in one of the bottles, the negative pressure caused by it is indicated by the difference in level of the clove oil in the limbs of the manometer.

The volume of gas, X , absorbed when a measured difference of pressure, P , is established is:

$$\left(\frac{V}{P} + A\right)$$

where V = volume of air in each bottle

A = cross-section area of manometer tube

P = barometric pressure in mm. of clove oil

Y = difference of levels of manometer liquids

2. *Method.*—In the following experiments 0.0288 gm. of dialuric acid (twice recrystallized) was weighed into one of the absorption bottles, dissolved in 2.5 cc. of doubly distilled water, and the whole neutralized to amphoteric reaction to litmus with a solution of redistilled ammonium hydroxide. This required 2.44 cc. of a solution containing 8 cc. of concentrated ammonium hydroxide in 1 liter. 10 cc. of distilled water were placed in the other absorption bottle.

Both bottles were then placed on their stoppers on the manometer, it being seen that they fitted firmly. The concentration of hydrogen ions in the solutions to be oxidized was controlled by standard buffer solutions. 5 cc. of buffer solution were introduced into the bottle containing the dialuric acid solution and the stopper firmly replaced.

Prior to this the levels of the manometer tubes have been read. The two stop-cocks are closed and the time noted. The manometer and absorption bottles were shaken vigorously throughout the experiment by a shaking apparatus. The bottles were kept immersed during the experiment in a water bath at practically room temperature. The temperature was recorded in all cases and the difference in level in the manometer tubes was read at 5 minute intervals as long as there was further absorption. The barometric pressure was also recorded for each experiment.

The buffer solutions used were those described by Clark (13). The following solutions were used for composing mixtures giving the different pH values.

pH	
1.2-2.2	0.2 M KCl + 0.2 M HCl.
2.4-3.8	0.2 " KH phthalate + 0.2 M HCl.
4.0-5.6	0.2 " " " + 0.2 " NaOH.
5.8-6.8	0.2 " KH_2PO_4 + 0.2 M NaOH.
7.0	0.05 " borax + 0.2 M boric acid containing 0.05 M NaCl.
7.2-9.2	0.2 " boric acid in 0.1 M NaOH + 0.1 M HCl.
9.4-12.0	0.2 " " " " 0.1 " " + 0.1 " NaOH.

As it was thought that the oxidation of dialuric acid might be catalyzed by small amounts of iron, precautions were taken to exclude, as far as possible, any trace of iron from the reaction mixture. Certain of the buffer solutions did, however, contain small amounts of iron and all of these solutions were tested for their iron content, which is tabulated below.

Dialuric acid.....	None.	
Dilute NH_4OH (8 cc. per liter).....	"	
0.2 M KCl	"	
0.2 ' HCl	0.000001	per cent.
0.2 ' KH phthalate	0.000003	" "
0.2 ' NaOH	0.000002	" "
0.2 ' KH_2PO_4	0.000005	" "
0.05 ' borax.....	None.	
0.2 ' boric acid.....	0.000001	per cent.
0.2 ' " " in 0.1 M NaOH	0.0000015	" "

The results of the experiments, with a variation in pH values from 1.0 to 12.0, are given in Tables I and II. The values for the negative pressure produced in the manometer by absorption represent a mean of three consecutive determinations, a slight variance being due to the difficulty in reading the manometer levels during the first part of the determinations, during which, in some cases, the oxidation was quite rapid.

From these figures an attempt was made to obtain a velocity constant for each of the different pH values by the formula:

$$= \left(\frac{1}{t} \right) \log \frac{C_b(C_a - C)}{C_a(C_b - C)}$$

The constants, however, usually diminished from the beginning to the end of the experiment except in the case of the very rapid oxidation at pH 7 and close to it, such as pH 6.4 (see Table II). This indicates that we have not yet taken account of all parts of the reaction, or that side reactions occur. The fact that the oxidation does not go to an end, except at pH 7, is also unaccounted for. However the mean or average constant at each pH gives the best means of comparison of the rate of oxidation which we have at present and accordingly they are used here (Table II).

TABLE I.

Absorption of Oxygen at Different pH Values.

pH.....	1.0	2.0	3.0	4.0	5.0	6.0	6.4	6.8	7.0	7.5	8.0	9.0	10.0	12.0
Time shaken.	Absorption, mm. difference of manometer level.													
min.														
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	38	51	56	85	98	45	44	30.5	131	45	92	94	114	144
10	64	96	124	157	180	120	83.5	54	240	65	138	145	167	227
15	90	134	144	188	210	167	134	72	274	80	155	168	194	267
20	106	151	158	216	222	185	159	81	304	85	164	187	211	294
25	117	161	166	228	235	203	174	90		88	171	194	224	306
30	124	166	171	232	241	212	178	98.5		90		198	232	312
35	131	169	173	234	242	226	178	99		91		205	239	
40	136	170	173	234	242	230	178		330	91		207	245	316
45	137	170	173	234	242	230	178	101	332	91	186	207	246	
50	137	170	173	234	242	230	178	101	332		186	207	246	319
60	137	170	173	234	242	230	178	101	332	91	186	207	246	320
Temperature, °C....	24.7	26.2	27.0	23.1	26.4	25.0	25.0	25.0	25.0	24.2	25.2	25.2	25.1	25.2
Barometer, mm. Hg.	745.2	743.6	744.8	746.4	740.3	742.6	748	742.8	740.7	750.8	751	746.1	746.1	740.0
Total volume O ₂ absorbed, cc.....	0.442	0.549	0.559	0.756	0.782	0.742	0.574	0.326	1.083	0.283	0.601	0.668	0.794	1.044
Per cent of total oxidation when absorption ceased.	40.0	49.5	50.3	68.1	70.4	66.9	51.7	29.3	99.1	26.4	54.1	60.2	71.5	94.0

TABLE II
Velocity C_i K , Oxidation

Time.	Values of K at various pH values.													
	1.0	2.0	3.0	4.0	5.0	6.0	6.4	6.8	7.0	7.5	8.0	9.0	10.0	12.0
min.														
5	0.0071	0.0084	0.0092	0.016	0.018	0.014	0.010	0.0072	0.047	0.010	0.013	0.013	0.017	0.058
10	0.0065	0.0083	0.0091	0.016	0.017	0.013	0.013	0.0064	0.048	0.0077	0.012	0.012	0.015	0.047
15	0.0063	0.0080	0.0089	0.016	0.018	0.014	0.011	0.0058	0.043	0.0065	0.010	0.011	0.016	0.043
20	0.0058	0.0078	0.0088	0.015	0.017	0.013	0.018	0.0050	0.048	0.0052	0.011	0.011	0.015	0.038
25	0.0057	0.0075	0.0088	0.015	0.017	0.013	0.010	0.0041		0.0043	0.0086	0.011	0.016	0.035
30	0.0055	0.0074	0.0087	0.015	0.016	0.012	0.009	0.0038		0.0037		0.011	0.015	0.029
35	0.0054		0.0084	0.014	0.014	0.013		0.0037		0.0032		0.010	0.013	
40	0.0054	0.0072				0.011		0.0031	0.047			0.0098	0.014	0.033
45	0.0052							0.0029	0.043		0.0078		0.014	0.028
50														
Mean K	0.0059	0.0077	0.0088	0.015	0.017	0.013	0.012	0.0046	0.046	0.0059	0.010	0.011	0.015	0.037

$$K = \left(\frac{1}{t} \right) \log \frac{C_b(C_a - C)}{C_a(C_b - C)}, \text{ from the velocity equation } \frac{dC}{dt} = K(C_a - C)(C_b - C)$$

C_a = concentration of dialuric acid

C_b = concentration of oxygen

C = concentration of alloxantin

DISCUSSION.

Fig. 1 shows comparatively the variation in the rate of absorption at different concentrations of hydrogen ions. In Fig. 1 the concentration of hydrogen ions is plotted on the abscissa, each division of which, passing from left to right, diminishes the concentration 10 times. The ordinates represent the mm. of pressure

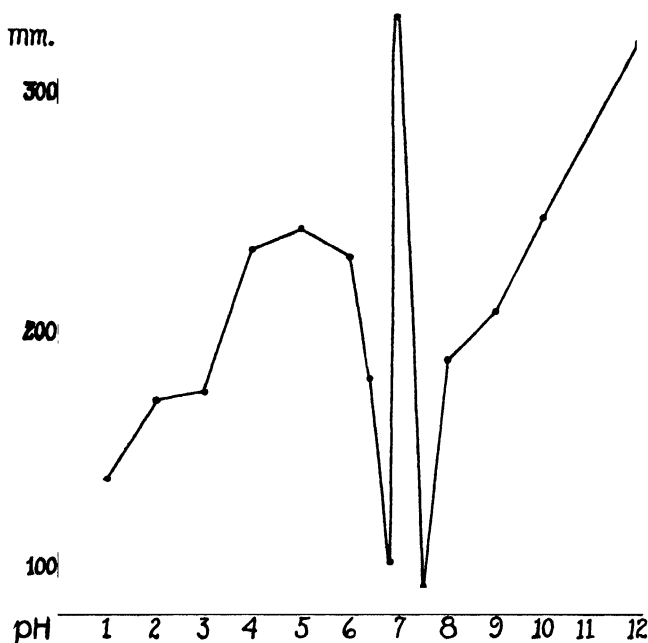


FIG. 1. Variation of oxygen absorption with the pH values. The abscissas show pH values, the ordinates negative pressure readings after 60 minutes absorption.

of clove oil due to the absorption of oxygen from the bottles after 60 minutes of shaking. In Fig. 2 the variation of the velocity constants with a change in pH values is shown. The ordinates represent the velocity constants and the pH values are plotted on the abscissa. The average constant for each determination was used for plotting.

It will be seen from Figs. 1 and 2 that this oxidation is extraor-

dinarily dependent upon the acidity and alkalinity of the solution. The spontaneous oxidation of dialuric acid takes place with appreciable rapidity both in acid and in alkaline solution but with quite extraordinary rapidity and completeness at the point of neutrality. On the acid side the absorption is greatest under the conditions of the experiment between the limits of pH 6 and 3. The maximum absorption lies at about pH 5, decreasing gradually, as the acidity increases, to pH 4, then somewhat rapidly to pH 3, gradually decreasing to pH 2, and then falling to pH 1. On the other side of pH 5 there is a decrease in the amount of absorption, gradually

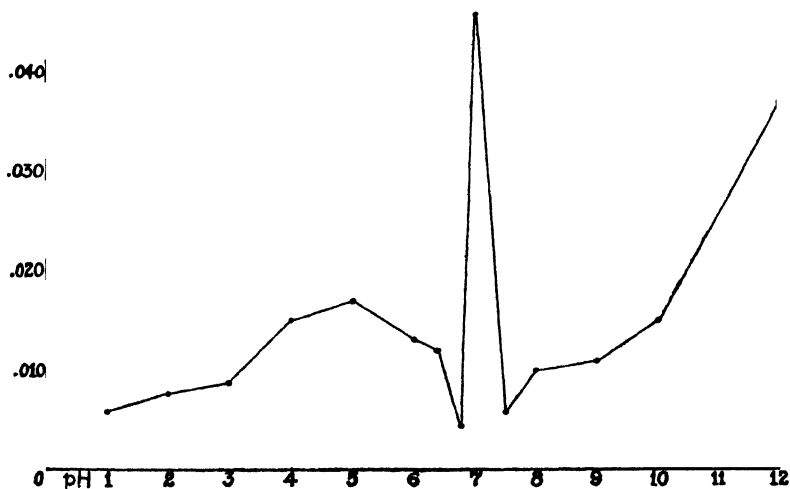


FIG. 2. Variation of the mean reaction constants with pH. The abscissas show pH values, the ordinates mean reaction constants.

to 6, and rapidly to pH 6.4 and 6.8, the lowest point on the acid side. From pH 6.8 there is a phenomenal increase in absorption to pH 7, at which point it is higher than at any other during the entire series of experiments. As soon as neutrality is passed the absorption falls to pH 7.5, its lowest point on the alkaline side and even the lowest rate found. On the alkaline side the absorption increases from pH 7.5 to 12, the most alkaline solution tested.

One point for consideration in an attempt at an explanation of the variation in rate of absorption is the presence of various metal salts in the buffer solutions and in the original constituents. It

was thought that the oxidation of dialuric acid might be accelerated by the presence of small amounts of iron and other metals, as is the oxidation of cysteine, as shown by Mathews and Walker (14).

The dialuric acid was free from iron, as was the ammonium hydroxide used to neutralize its acidity when dissolved. At a hydrogen ion concentration of $N 10^{-7}$, the buffer used was a mixture of 0.05 M sodium borate and 0.2 M boric acid containing 0.05 M sodium chloride. The borax solution was free from iron and the boric acid salt solution contained 0.000001 per cent. The buffer mixture for this pH (7.0) in which oxidation was most rapid was less contaminated by iron than that for any other hydrogen ion concentration. The buffers for a range of pH 5.8 to 6.8, made of 0.2 M potassium dihydrogen phosphate and 0.2 M sodium hydroxide, contained iron in quantities of 5 to 7 parts per million and the fact that the absorption was accelerated with increasing amounts of phosphate solution used to increase the acidity might indicate catalysis by the iron. The buffers for pH 4.0 to 5.6 contained 3 parts per million in the potassium hydrogen phthalate solution and 2 parts per million in the sodium hydroxide. Solutions used between pH 2.4 to 3.8 and pH 1.0 to 2.2 contained 3 parts per million in the phthalate and 1 part per million in the hydrochloric acid for the former and none in the potassium chloride solution, with 1 part per million in the hydrochloric acid for the latter. Above pH 7.0 the buffers were more pure; the borax solution was free from iron, the boric acid solution contained 1 part per million, and the boric acid-sodium hydroxide solution used for the range pH 9.4 to 12.0 contained 1.5 parts per million. At these last hydrogen ion concentrations there was a pronounced increase in absorption. The results are such as to indicate that the spontaneous oxidation is not dependent on the presence of iron.

It is possible that other unknown metal salts in the solutions might have an accelerating or retarding action on this oxidation. Richardson and Cannan (6) state that their results were not closely reproducible, there being, apparently, uncontrolled catalytic effects and that these did not appear to be of the heavy metal type, since the addition of HCN was without notable effect. The catalytic peculiarities of the metals toward this oxidation are to be considered in a further investigation.

The fact that the reactions did not come to an end, there being

only partial oxidation in all of the cases except that at neutrality, is also unexplained. Whether this is due to a reversal of the reaction under the conditions observed, or to some other factor is not known but will provide material for further research.

SUMMARY.

1. Dialuric acid oxidizes itself spontaneously by atmospheric oxygen at 20 to 25° at a rapid rate, passing over into alloxan.

2. This oxidation is extremely sensitive to the reaction of the medium, the maximum oxidation being obtained at a concentration of hydrogen ions of about $N 10^{-7}$ and falling to a minimum at pH 6.8 and 7.5, from which it rises in each direction. On the acid side of neutrality the oxidation is best between pH 3.0 to 6.0. On the alkaline side the speed of reaction increases with an increase in hydroxyl ions from pH 7.5.

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THE EFFECT OF HEMORRHAGE ON THE ACID-BASE EQUILIBRIUM OF THE BLOOD.

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After severe hemorrhage, alterations in the acid-base equilibrium of the blood have been frequently observed. Working in this laboratory, Bennett (1) found increased hydrogen ion concentration of arterial blood $\frac{1}{2}$ hour and 4 hours after hemorrhage and Riegel (2) showed that these changes were associated with an accumulation of lactic acid. As Bennett's work was being completed Gesell and Hertzman (3) reported that, in anesthetized animals, the manganese dioxide electrode indicated that arterial blood becomes more alkaline after hemorrhage, though after a time a swing toward the acid may occur. On account of the possibility of obtaining erratic results with the manganese dioxide electrode, especially in the presence of abnormal metabolic products, we thought it desirable to try to correlate Bennett's and Gesell's findings by further studies with the hydrogen electrode. Work was started in the fall of 1926. The appearance of the paper by Gesell and Hertzman (4), showing poor agreement between the manganese dioxide and quinhydrone electrodes after hemorrhage and reinjection and after sodium cyanide injection, emphasized the need for further study of the conditions by the use of the hydrogen electrode. The experiments reported below were carried out during 1927. Unusual circumstances have caused delay in preparing the data for publication.¹

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¹ A report of this work was made before the Physiological Society of Philadelphia on November 21, 1927, and an abstract published in their proceedings (*Am. J. Med. Sc.*, 175, 149 (1928)).

Since the work reported below was begun, papers have appeared from Gesell's laboratory dealing with the effects of hemorrhage on the hydrogen ion concentration of arterial and venous blood. Bald (5) studied anesthetized and decerebrate dogs, using Hastings' modification of Cullen's colorimetric method. After hemorrhage he observed an increased pH of blood from 0.1 to 0.3 above the value obtained at the beginning of the experiment with bleedings of 1 or 2 per cent of the body weight. While the colorimetric methods have been shown by Bennett (1) and Johnston (6) to be untrustworthy, the variations reported by Bald are outside the probable errors. A further report was made later by Hertzman and Gesell (7) in which manganese dioxide electrode variations were checked in a few cases with hydrogen electrode determinations. This report demonstrated satisfactorily that the transient increase in alkalinity of arterial blood, which was indicated by the manganese dioxide electrodes to occur after hemorrhage, was real. All these observations were made on anesthetized animals. As our experiments were carried out on unanesthetized animals, it is of interest to compare the types of response in the two cases. Besides determination of the pH changes, analyses were also made of the CO_2 and lactic acid of blood, and the effect of repeated hemorrhage was studied.

EXPERIMENTAL.

Normal unanesthetized dogs were used throughout the experiments. Blood was drawn from the femoral artery through a cannula which was put in place, with cocaine as a local anesthetic. In this way hemorrhage could be carried out rapidly. An initial sample of blood was drawn for analysis and additional bleeding made rapidly. For heavy hemorrhage the total of the two portions amounted to 2 to 3 per cent of the body weight. We have designated the hemorrhages light when they amounted to less than 2 per cent of the body weight. A second sample for analysis was often taken a few seconds after the heavy hemorrhage had been completed. In most of the experiments to be reported the three bleedings were made within 3 to 6 minutes. For all analyses except lactic acid, blood was allowed to flow under oil into centrifuge tubes. After clotting, the oil was replaced by paraffin, the blood was centrifuged, and the serum was transferred to a tonom-

eter. In the experiments reported, the whole experimental procedure was carried out without exciting the animal.

The pH was determined by means of the hydrogen electrode at 37° (8) and the CO₂ by Van Slyke's (9) volumetric apparatus. The lactic acid was determined by Clausen's (10) procedure, with whole blood to which oxalate and fluoride had been added.² Analyses were completed in about 2 hours. Free CO₂ or H₂CO₃ was calculated from the total CO₂ by means of the following formula derived from the formulas summarized by Austin *et al.* (11).

$$\text{H}_2\text{CO}_3, \text{ mm per liter} = \frac{\text{CO}_2}{10^{\text{pH} - \text{pK}'} + 1}$$

where CO₂ is expressed in mm per liter and pK' = 6.10. The calculations were checked against the line chart of Van Slyke and Sendroy (12). Combined CO₂ was obtained by subtracting the free CO₂ from the total. Alkaline reserve was calculated as combined CO₂ at normal pH, on the assumption that $\frac{d\text{BHCO}_3}{d\text{pH}} = 25$ on the 1st day of the hemorrhage and 17 on the 2nd day when the blood contained a lower hemoglobin concentration. The errors of this procedure may be large in three or four instances where the pH change was great but can hardly influence the discussion.

The results of our experiments will be found in Tables I and II.

We have twelve observations of the pH of blood 1 or 2 minutes after a heavy, rapid hemorrhage carried out on dogs presumably normal. No change from the normal was found in six experiments, while the others showed increased alkalinity varying from 0.05 to 0.13 pH. Two observations 6 minutes after heavy hemorrhage showed blood more acid than normal. The pH tended to be further lowered 15 minutes after hemorrhage. Blood of Dog 27 was more alkaline than normal 20 minutes after a moderate hemorrhage (2.5 per cent of body weight). After hemorrhage of less than 2 per cent, no changes outside the experimental error were encountered.

These isolated observations are far from ideal in attempting to determine the alterations taking place after hemorrhage, because

² The lactic acid determinations were carried out by Mr. E. G. Ball.

TABLE I.
Changes in Blood Composition Due to Hemorrhage.

	Date.	Time interval.	Hemor- rhage.		pH	mm per liter.			
						H ₂ CO ₃	BHCO ₃	BHCO ₃ at initial pH.	Lactic acid.
Dog 3, 15.0 kilos.	1927 May 16	60 sec.	cc. 100	per cent body weight 0.67	7.41*	0.9	19.2	19.2	2.5
		60 "	300	2.00					
		60 "	100	0.67	7.38*	0.8	15.5	14.8	2.6
		15 min.							
		60 sec.	100	0.67	7.14	0.9	9.5	2.8	5.6
Dog 13, 22.8-23.8 kilos.	Apr. 5	60 sec.	150	0.66	7.38				
		120 "	525	2.30					
		60 "	150	0.66	7.36*				
	June 16	45 "	100	0.42	7.40*	1.1	20.7	20.7	2.4
		80 "	650	2.73					
		50 "	100	0.42	7.40*	1.0	20.2	20.2	2.3
		10 min.							
		45 sec.	100	0.42	(1.0)	(19.5)			2.8
		4 hrs.							
	June 17	60 sec.	100	0.42	7.40*	0.9	19.0	19.0	2.2
		30 "	100	0.42	7.41	1.1	22.7	22.9	2.0
		150 "	350	1.47					
		120 "	100	0.42	7.42	0.9	18.7	19.0	2.0
		10 min.							
		90 sec.	100	0.42	7.36	0.8	14.6	13.9	5.7
Dog 14, 17.2kilos.	Apr. 7	60 sec.	150	0.87	7.40				
		120 "	300	1.74					
		60 "	150	0.87	7.49				
Dog 16, 15.2 kilos.	Apr. 14	60 sec.	150	0.99	7.43				
		60 "	320	2 10					
		60 "	150	0.99					
	Apr. 15	60 "	150	0.99	7.46	0.9	21.6	22.1	
		120 "	300	1.97					
		60 "	150	0.99	7.62	0.4	13.7	16.9	
Dog 17, 19.1 kilos.	Apr. 21	60 sec.	150	0.79	7.45	1.0	21.8	21.8	
		120 "	475	2.48					
		60 "	150	0.79	7.58	0.5	15.9	19.1	
	Apr. 22	60 "	150	0.79	7.41	1.0	19.9	19.2	2.0
		120 "	450	2.36					
		60 "	150	0.79	7.74	0.3	11.7	16.6	2.4

Determinations were made in duplicate except where indicated by an asterisk.

Parentheses indicate that BHCO₃ has been calculated from the total CO₂, the value given for H₂CO₃ being assumed.

TABLE I—Continued.

	Date.	Time interval.	Hemor- rhage.		pH	mm per liter.			
						H ₂ CO ₃	BHCO ₃	BHCO ₃ at initial pH.	Lactic acid.
Dog 18, 27.7 kilos.	1887		cc.	per cent body weight					
	Apr. 26	60 sec.	150	0.54	7.36	1.2	20.7	20.7	
		120 "	650	2.35					
	Apr. 27	60 "	150	0.54	7.41	0.7	14.2	15.4	
		15 min.							
		60 sec.	150	0.54					4.3
		60 "	150	0.54	7.43*	0.9	18.2	19.4	3.1
		120 "	650	2.35					
		60 "	150	0.54	7.54	0.4	11.3	14.4	
		15 min.							
		60 sec.	150	0.54	7.15	0.5	5.0	1.4	
Dog 19, 18.4 kilos.	May 2	60 sec.	150	0.82	7.41*	1.0	21.0	21.0	2.0
		6 min.	475	2.58					
		60 sec.	150	0.82	7.35	1.0	17.7	16.2	2.5
		15 min.							
		60 sec.	150	0.82	7.25	0.9	13.2	9.2	6.2
Dog 21, 17.0 kilos.	May 5	60 sec.	150	0.88	7.46	1.0	22.4	22.4	1.2
		60 "	240	1.41					
		60 "	150	0.88	7.45*	0.9	19.5	19.3	
		15 min.							
		60 sec.	150	0.88	7.26	1.1	15.4	10.4	2.3
Dog 23, 13.6 kilos.	May 17 " 18	18 min.	610	4.48					
		60 sec.	100	0.74	7.38	1.0	19.0	19.0	2.1
		60 "	300	2.20					
		60 "	100	0.74	7.54	0.5	13.7	16.4	3.0
		5 min.							
Dog 24, 16.2 kilos.	June 7	60 sec.	100	0.74	7.24	0.5	7.3	4.9	
		30 sec.	100	0.62	7.40	1.2	23.4	23.4	3.0
		60 "	300	1.86					
		30 "	100	0.62	7.45*	0.9	20.7	21.9	3.1
		5 min.							
	June 8	30 sec.	100	0.62	7.23	1.4	18.9	14.7	3.6
		30 "	100	0.62	7.42	1.1	21.5	21.8	3.1

TABLE I—Continued.

	Date.	Time interval.	Hemor- rhage.		pH	mm per liter.			
						H ₂ CO ₃	NaHCO ₃	BHCO ₃ at initial pH.	Lactic acid.
Dog 25, 12.0 kilos.	1987 June 22	25 sec.	100	0.83	7.35*	1.1	19.2	19.2	3.1
		25 "	180	1.50					
	June 23	55 "	100	0.83	7.46*	0.7	15.8	18.5	2.3
		15 min.							
		8 "	100	0.83	7.27*	0.8	10.9	8.9	5.9
		12 sec.	50	0.42	7.34*				3.3
		31 "	150	1.25					
		18 "	50	0.42					3.6
		10 min.							
		30 sec.	50	0.42	7.44*				6.2
		20 min.							
		20 sec.	50	0.42	7.10*				11.9
Dog 26, 18.5 kilos.	June 29	32 sec.	100	0.54	7.32	1.2	20.5	20.5	2.5
		85 "	440	2.38					
		70 "	100	0.54	7.27Q	1.2	16.6	15.4	2.4
		15 min.							
	June 30	45 sec.	100	0.54	7.22*	1.1	15.3	12.8	4.5
		4 min.	100	0.54	7.32*	1.2	20.7	20.7	2.9
		15 "							
		35 sec.	100	0.54	7.33*	1.2	20.7	20.9	3.0
		100 "	340	1.84					
		90 "	100	0.54	7.43*	0.7	15.7	17.6	3.1
		14 min.							
		65 sec.	100	0.54	7.08	0.6	5.9	1.8	12.4
		10 min.							
		80 sec.	100	0.54	7.10*	0.3	3.2	0	16.5
Dog 27, 10.0 kilos.	July 14	25 sec.	100	1.00	7.35*	1.3	24.3	24.3	2.0
		60 "	150	1.50					
		15 min.							
	July 16	60 sec.	100	1.00	7.36*	1.2	22.3	22.5	2.9
		40 "	100	1.00	7.33*	1.3	23.8	23.5	2.5
		50 "	150	1.50					
		20 min.							
		50 sec.	100	1.00	7.39	0.8	16.3	17.0	5.3

Q indicates quinhydrone determination.

TABLE I—*Concluded.*

	Date.	Time interval.	Hemor- rhage.		pH	mm per liter.			
						H ₂ CO ₃	BHCO ₃	BHCO ₃ at initial pH.	Lactic acid.
	1927		cc.	per cent body weight					
Dog 28, 13.0 kilos.	July 19	30 min.	250	1.92					
	" 20	3 hrs.	200	1.54					
	" 22	30 sec.	50	0.38	7.30	1.2	20.5	20.5	
		30 "	50	0.38	7.30	1.2	20.5	20.5	
		30 min.							
		30 sec.	50	0.38		(1.2)	(20.0)		
		2 hrs.							
		30 sec.	50	0.38	7.23*	1.4	18.8	17.6	
Dog 33, 22.0 kilos.	Nov. 10	30 sec.	100	0.45	7.32	1.2	20.1	20.1	
		90 "	500	2.28					
		30 "	100	0.45	7.33	1.1	18.9	19.1	
	Nov. 11	60 "	150	0.45	7.37	1.2	21.4	22.6	
		90 "	500	2.28					
		60 "	50	0.23	7.52	0.7	18.5	21.9	

of the rapid change which may occur in the intervals between samplings. There is also a disadvantage in taking the large samples necessary for the studies we were making. There appears to be a quantitative difference, however, between our experiments and those carried out in Gesell's laboratory on anesthetized and decerebrate animals. Bald (5) and Hertzman and Gesell (7) report (a) greater alkaline swings after hemorrhage, (b) a longer interval during which the arterial blood is more alkaline than normal, and (c) alkaline swings after smaller hemorrhages than those found by us to be effective. Hemorrhages of less than 1 per cent of the body weight usually seem to result in alkaline swings in Gesell's dogs, whereas we have not observed alkaline changes after hemorrhages of 2 per cent of the body weight. It is possible that we may have missed temporary swings on account of our infrequent sampling but we doubt if that is the whole explanation. The differences may be ascribed most probably to the differences in response of anesthetized and unanesthetized animals.

Date.	Time interval.	Hemorrhage.		pH	mm per liter.				Respiration per min.		Notes on respiration data.
		cc.	per cent body weight		H ₂ CO ₃	BHCO ₃	BHCO ₃ initial pH	Lactic acid.	Liters.	Rate.	
Dog 11, 19 kilos.											
	25 sec.	100	0.5	7.36	1.3	24.0	24.0		2.1	13	Data obtained during 5 min.
	55 "	450	2.4						1.5	13	period preceding 1st hemorrhage.
	39 "	100	0.5	7.47	0.8	19.4	22.1	2.7	2.4	13	
	15 min.										Data obtained during 15 min. interval between 3rd and 4th hemorrhages.
	60 sec.	100	0.5	7.31				2.8	16		
								2.7	18		
											Data obtained immediately after last hemorrhage.
									3.5	20	
									3.8	17	

Dog 30, 35 kilos.	Aug. 17											Aug. 18	
35 sec.	100	0.3	7.33*	1.3	22.4	22.4	3.8	Data obtained during 2 min. period preceding 1st hemorrhage.					
60 "	750	2.1					3.8	Data obtained in consecutive 1 min. intervals starting with initiation of 1st hemorrhage.					
40 "	100	0.3	7.35*	1.1	20.0	20.5	3.6	16					
							2.7	16					
25 min.							4.3	17	Average of 5 readings taken during interval between last 2 hemorrhages.				
							4.9		Reading taken during last hemorrhage.				
25 sec.	100	0.3	7.30*	1.0	16.1	15.4	5.4		5 min. after last hemorrhage.				
							4.4	15	15 min. later.				
							3.7	12					
							3.1	12	Average of 3 readings taken in 3 min. period preceding 1st hemorrhage.				
30 sec.	50	0.1	7.32*	1.2	20.7	20.5	3.4	12	Data obtained in consecutive 1 min. intervals starting with initiation of the 1st hemorrhage.				
150 "	1075	3.1					3.5	14					
							4.1	14					
30 "	50	0.1	7.44*	0.6	13.9	15.8	3.8	14					
15 min.							3.9	15	1 reading made during period between last 2 hemorrhages.				
30 sec.	50	0.1	7.00*	1.0	8.1	2.5	1.9	17	Data obtained during last hemorrhage. Dog in bad condition; died few minutes later.				

* Determinations were made in duplicate except where indicated by an asterisk.

When hemorrhages were repeated on the same animals the day following a heavy hemorrhage, greater changes were observed. A minute after a heavy hemorrhage the blood was always found to be alkaline (seven observations varying from $+0.08$ to $+0.33$ pH). A swing to the acid side of normal occurred within 5 to 15 minutes as on the 1st day. Our observations of a decreased pH of blood developing a few minutes after heavy hemorrhage and persisting for a considerable time confirm the findings of Bennett.

We assume that the low red cell concentration (hemoglobin) in the blood the day after a heavy hemorrhage accounts for the greater pH changes, but the mechanisms causing the change may be several. It would seem that the diminished buffer capacity of the blood is the main factor concerned in the exaggerated alkaline swing. Overaeration of the blood in the lungs, due to increased pulmonary ventilation, would result in a greater pH change of blood the lower the hemoglobin concentration. The transport of O_2 and CO_2 being less effective than normal, there might result a more rapid piling up of acid in the respiratory center with greater respiratory response and consequent greater overaeration of the blood.

Hemorrhage often failed to alter the breathing appreciably. However, the rate of respiration increased in some experiments (15 to 60 per minute in one experiment and 19 to 44 in another) and at times the respirations were deeper. When this was noticeable, the blood was found to be more alkaline immediately after the heavy hemorrhage. The pulse rate also increased. In two experiments, attempts were made to determine the minute volume of air breathed by having the animals breath into a mask connected with a Bohr meter (see Table II). These animals were made accustomed to the mask for several weeks before the experiments were carried out. In one instance (Dog 30, 35 kilos) a very large animal was used so that the withdrawal of blood for analysis would not constitute hemorrhage of appreciable magnitude. A most striking point of these experiments is the apparently slight increase in minute volume of air breathed necessary to increase the pH of the arterial blood.

While the lowered pH of blood persists for a number of hours after a heavy hemorrhage, the reaction is normal on the following

day. We have nine observations on the pH of blood immediately before and 24 hours after heavy hemorrhage with no significant variation from the normal. Bennett obtained blood more alkaline than normal on the day following a heavy hemorrhage in some experiments. Considering the ease with which the pH may be caused to increase in these anemic dogs, it is our impression that the time spent by Bennett in collecting a large blood sample through needles was sufficient to allow the blood to become more alkaline by slight overventilation of the animal.

The concentration of total CO_2 of the blood diminished in all experiments except where the hemorrhage was slight. The decrease in total CO_2 may result from a number of different causes. We have data which will permit a study of several of these; namely, alterations in the concentrations of free CO_2 ($\text{H}_2\text{CO}_3 + \text{CO}_2$), of combined CO_2 (BHCO_3), and of lactic acid.

The free CO_2 was found to be decreased 1 minute after hemorrhage. The greatest decreases were associated with the greatest increases in alkalinity of the blood. This fact indicates that the increased alkalinity was due to the increased aeration of the blood. It is doubtful whether the decreased CO_2 transport observed by McGinty and Gesell (13) after hemorrhage could have been an appreciable factor at that time. A rise of free CO_2 above the minimum value was often observed when the blood pH was low.

The combined CO_2 was also diminished immediately after hemorrhage. Care must be taken in considering these changes, because when the blood becomes more alkaline the base-binding power of the blood proteins increases. They thus act as acids and react with the BHCO_3 , releasing CO_2 and combining with the base. Calculation of the base which would be combined with CO_2 at normal pH corrects for the changed combining power of proteins and gives a more accurate idea of the alterations in blood alkali due to lactic acid neutralization and other causes. These calculated values designated for convenience as alkaline reserve, show a decrease after hemorrhage. Decreases in combined CO_2 and alkaline reserve also occur when the blood does not become more alkaline. In some instances such as Dog 13, June 16, a steady drop in combined CO_2 occurred during a period of 4 hours with little change in pH. Lactic acid did not account for the decrease.

The lactic acid concentration in the blood appears to rise only slowly. One observation 6 or 7 minutes after a heavy hemorrhage showed a value slightly above the normal. 15 minutes after hemorrhage the lactic acid had risen considerably in most experiments.

The greatest change in alkaline reserve and lactic acid occurred on the 2nd day of hemorrhage. Dog 26, June 30, shows the most extreme changes which were observed. On the 2nd day of hemorrhage the lactic acid rose to 16.5 mm per liter (148 mg. per 100 cc.) and the pH and total CO_2 fell to 7.10 and 3.5 mm per liter respectively. Had the pH of the blood remained normal, the combined CO_2 would have fallen to zero.

With the rise in lactic acid it is natural to expect diminution in the alkaline reserve. However, in practically all instances the increase in lactic acid accounted for only a small part of the decrease in the alkaline reserve. The calculation of alkaline reserve, when there is an appreciable change in the pH of blood, is subject to considerable error and the lactic acid method is far from ideal, but the differences between the decreases in alkaline reserve and increases in lactic acid are so great that we feel that the observation is real. As much as 13 mm of base was unaccounted for in one experiment and the discrepancy was frequently 4 to 10 mm.

The reason for the discrepancy between the fall in alkaline reserve and the rise in lactic acid is not apparent. Koehler, Brunquist, and Loevenhart (14) observed the same phenomenon in studying asphyxia due to breathing atmospheres low in oxygen. They state that the low total CO_2 of blood in anoxemia is not due to an accumulation of lactic acid and "acetone bodies" but give no data. They suggest that the phosphates may be increased.

The loss of bicarbonate unaccounted for by lactic acid neutralization suggests a consideration of the unique theory proposed by Henderson (15) and developed by Haggard and Henderson (16, 17). They proposed that the lowering of the total CO_2 content of the blood after hemorrhage and other experimental means of producing asphyxia is due to acapnia and not to the development of metabolic acids. They believed that the overventilation which accompanies asphyxia reduces the concentration of free CO_2 of the blood, produces an increased pH, and, as a result, there occurs a diffusion of alkali from the blood, thus reducing the BHCO_3 .

concentration of the blood. Any increase in H_2CO_3 concentration, they assumed, would cause the alkali to return to the blood.

Our observations as well as those of Riegel (2) show that lactic acid may accumulate in the blood after hemorrhage and may account for an appreciable part of the loss of alkaline reserve. In attempting to apply the acapnia theory of Henderson and Haggard to explain the loss of alkaline reserve unaccounted for by lactic acid accumulation, the following observations should be noted. Whereas the greatest increase in pH 1 minute after hemorrhage is associated with the greatest reduction in free H_2CO_3 , this maximum change is not associated with the greatest loss in alkaline reserve. In fact the two blood samples, showing shifts toward the acid side actually had the greatest decrease in alkaline reserve 1 minute after hemorrhage. Furthermore, still greater reductions in alkaline reserve unaccounted for by lactic acid accumulation were observed 15 to 20 minutes after hemorrhage, when the blood was practically always more acid than normal and the lactic acid concentration high. The acapnia theory does not appear to offer an adequate explanation of these changes.

It is improbable that the lipemia³ after hemorrhage with a possible increase in lipid phosphate or free fatty acid might account for the binding of base. The lipemia probably develops slowly and is very obvious on the day following a heavy hemorrhage, when the alkaline reserve has returned to normal. As we have evidence to indicate that the common inorganic ions are not concerned in the decreased alkaline reserve, we are led to assume that other acid metabolic products should be considered.

Certain correlations may be made between our observations and the physiological changes known to occur after hemorrhage. After severe hemorrhage, such as occurred in most of our experiments, sufficient interference with the circulation results so that a condition of oxygen-lack develops in the tissues. A transient overaeration of the blood is quickly followed by a rapid swing in

³ Bloor (18) states that whereas it is easy to produce lipemia in rabbits, by hemorrhage, it was found impossible to produce lipemia in dogs or even change appreciably the blood lipid values. We have often observed considerable milkiness in the serum of dogs the day after a heavy hemorrhage and occasionally increased turbidity during a day when several bleedings were carried out.

blood pH to below the normal value. With developing anoxemia, lactic acid slowly accumulates in the blood, together with possibly other compounds of acidic nature which accumulate with greater rapidity. A diminished pH, lowered alkaline reserve, and high lactic acid concentration persist until, by compensations such as constriction of the vascular bed and inflow of fluid, the circulation is gradually caused to improve. The restoration of blood volume seems to be the main factor associated with the disappearance of the anoxemia as indicated by the return to normal of the blood pH, alkaline reserve, and lactic acid concentration.

SUMMARY.

After severe hemorrhage in normal unanesthetized dogs, the arterial blood tended to become first, more alkaline, then quickly more acid than normal. The changes are similar but apparently not as great as those observed by Gesell on anesthetized dogs. The increased pH appeared to be associated with and presumably due to a slight overventilation.

Greater changes of similar nature were observed after hemorrhage in dogs made anemic by previous bleeding.

The increased acidity of the blood was associated with an accumulation of lactic acid.

The lactic acid accumulation accounted for only a small part of the diminution of alkaline reserve which always occurred after hemorrhage.

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A SPECTROGRAPHIC STUDY OF CARBON MONOXIDE HEMOGLOBIN.

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PLATES 5 TO 7.

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Carbon monoxide hemoglobin is species-specific. This fact has been shown recently by immunological methods (1). This specificity may be due to differences in the prosthetic group of the molecule of this conjugated protein, in the histone (globin) fraction, or in both the prosthetic group and the globin. The antigenic differences suggest, but do not conclusively prove that the globin fraction is the chemically species-specific part of the compound since this is the protein part and the largest portion of the molecule. It is presumed that the visible absorption spectrum of the carbon monoxide hemoglobin is due primarily to the effect of the colored prosthetic group. Since pure carbon monoxide hemoglobin was available, a study of spectra of this material presented itself, with the possibility that the species-specific portion of the molecule might be made evident.

In 1868 Preyer (2) showed the differences in visible spectrum between oxyhemoglobin, carbon monoxide hemoglobin, and other hemoglobin derivatives. Doumer and Fourrier (3) and Barcroft ((4) p. 41) have also compared the oxyhemoglobin and carbon monoxide hemoglobin spectra. In 1876 Sorby (5) measured the two main visible field absorption bands of human (normal) hemoglobin, and of blood of *Planorbis corneus*. He found them to be as follows:

	Center of band.	
	$\mu\mu$	$\mu\mu$
Normal oxyhemoglobin.....	581	545
<i>Planorbis corneus</i>	578	542½

Sorby concluded from this difference that in the *Planorbis* occurred, not the same oxyhemoglobin as in human, but a modification of it. Gamgee ((6) p. 131) in 1880 repeated the measurements with Preyer and found that "the position of the bands in *Planorbis* as stated above really coincides almost exactly with that of the bands of oxyhemoglobin." Welker and Williamson (7) found "that there is not sufficient difference in absorption coefficients of the hemoglobin of various species to serve as a means of identification of the species. This finding confirms the conclusions of most of the previous investigators." They used pure hemoglobin in their investigation. In 1922 Vlès (8) found considerable differences in spectra between the various species. Suhrmann and Kollath (9) gave some spectrophotometrical results on plasma and blood corpuscles of rat and blood corpuscles of guinea pig and man. Their results on the blood corpuscles of rat, guinea pig, and man indicated differences in absorption spectrum, especially between that of rat and man. However, this work was done on the mixture of the several blood proteins, lipoids, etc.; no pure hemoglobin was used. Barcroft (4), using a Hartridge reversion spectroscope, claimed differences in the spectra of both oxyhemoglobin and carbon monoxide hemoglobin from the different species.

The oxyhemoglobin and carbon monoxide hemoglobin, a study of which by spectrographic methods is herein reported, were prepared by a previously described method (1). This method was essentially as follows: Oxyhemoglobin was first prepared by the Marshall and Welker aluminum hydroxide cream method (10). Oxalated blood was centrifuged to procure the red corpuscles. This corpuscular mass was washed several times with salt solution and then laked with distilled water. Aluminum hydroxide cream was then added and the mixture filtered in a refrigerator. Marshall and Welker have shown that the filtrate contains no protein other than the oxyhemoglobin. The aluminum hydroxide cream used was made by adding slowly and with constant stirring, a 1 per cent solution of ammonium hydroxide to a 1 per cent solution of ammonium alum until the reaction was slightly alkaline. The creamy precipitate formed was then washed several times by decantation, the supernatant liquid finally giving a negative ammonia test with Nessler's reagent. Carbon monoxide hemoglobin was made

by saturating the oxyhemoglobin with CO gas which was generated by adding formic acid to warm, concentrated sulfuric acid. The gas was twice washed with sodium hydroxide and water. Both the oxyhemoglobin and carbon monoxide hemoglobin were then recrystallized from absolute alcohol at 0° or below. The concentrations of the materials were chosen as the result of a trial and error process. That concentration giving a clear, contrasting complete spectrum photograph in the time indicated in Figs. 1 to 7 was considered suitable.

In the work herein reported light from a Kromayer mercury arc lamp, when the ultra-violet spectrum was studied, or a 500 watt Mazda lamp, when only the visible spectrum was of interest was passed through quartz cells of 1 cm. thickness which contained the material under examination. A Hilger quartz spectrograph was used, spectra being photographed on Eastman orthochromatic and panchromatic plates.

All of the figures accompanying this paper are negatives; that is, the darkened portions of the plates are those areas affected by transmitted light. On all plates are one or two comparison spectra transmitted through empty cells and a series of spectra transmitted through the same cells filled with the material being studied. In Fig. 1 the dark lines represent the discontinuous mercury vapor spectrum, with or without the absorbing substance inserted. A comparison of the optical density of the corresponding lines gives the time of exposure necessary to restore the original intensity of that particular wave-length, although the absorbing substance is inserted. In this way the dotted curve in Fig. 1 was obtained, approximately representing a transmission curve. Figs. 2, 3, 4, and 6 were not used for such quantitative results, but rather to give evidence of small variations of transmission in nearly identical substances. In Figs. 5 and 7 the transmission is more evident, but still less quantitatively shown, on the background of a continuous spectrum from the 500 watt Mazda lamp, which, however, does not extend as far into the region of the shorter wave-lengths as 300 $\mu\mu$.

The approximate transmission curve of carbon monoxide hemoglobin is shown in Fig. 1, sheep carbon monoxide hemoglobin being taken as an example. The absorption bands appear as follows: the far ultra-violet end absorption occurs at 240 $\mu\mu$, the far ultra-

violet absorption at $280\ \mu\mu$, and the violet at approximately $400\ \mu\mu$. Visible absorption also occurs at about 540 and $570\ \mu\mu$, but this is made more evident by using the more continuous spectrum from electric light bulb source or by optical observation. It appeared of interest to include human carbon monoxide hemoglobin among the different species compared, but no crystallized human carbon monoxide hemoglobin was available. Absence of any spectrum differences between crystallized and non-crystallized horse carbon monoxide hemoglobin was shown (Fig. 2). Exposures of 1, 10, and 100 seconds were used. The spectra taken on the same photographic plate are the same, although the one material was recrystallized four times.

Fig. 3 shows spectra of human, sheep, hog, and ox carbon monoxide hemoglobin. The exposures are 10 and 100 seconds each, and comparison spectra of 1 and 10 seconds are also shown. No difference in spectrum of the different species is evident.

The relative stability of carbon monoxide hemoglobin and oxyhemoglobin was also investigated. On Fig. 4 are shown spectra of freshly made carbon monoxide hemoglobin of the same four species as shown on Fig. 3 and also material made by passing CO through human oxyhemoglobin which had been kept in the refrigerator at 0° for $\frac{1}{2}$ year, and human carbon monoxide hemoglobin made 12 days previously from fresh oxyhemoglobin. The spectrum of the material made from the half year old oxyhemoglobin shows considerable difference from wave-lengths 360 to $450\ \mu\mu$. Either the product of denaturation of the oxyhemoglobin by long standing is incapable of combining with CO to form carbon monoxide hemoglobin or a different combination of carbon monoxide and hemoglobin is produced. No change in carbon monoxide hemoglobin after 12 days standing was indicated by its spectrum.

The fact that oxyhemoglobin changes upon standing at room temperature is also shown in Figs. 5 to 7. A water solution of oxyhemoglobin was dialyzed in a collodion bag against distilled water at room temperature. After a few hours enough of a brown protein had passed through the bag to color the dialysate solution. Fresh oxyhemoglobin solutions are red, or in very dilute condition yellowish red. No red colored material passed through the collodion membrane. In Fig. 5 photographs are shown of the visible

spectrum of both fresh hog oxyhemoglobin solution and the brown dialysate exposed for 2, 5, 20, and 100 seconds. Comparison spectrum exposures of 1 and 10 seconds are also included. In Fig. 6 is a photograph of spectra produced by the ultra-violet lamp of the same materials as in Fig. 5. Protein content of the oxyhemoglobin and dialysate solutions is the same, in Fig. 6, as determined by the Kjeldahl method. Differences in spectra are evident at wave-lengths of 250, 436, and 578 μ .

It has been repeatedly stated ((6) p. 109 (11-12)) that oxyhemoglobin in water solution changes to methemoglobin upon standing. These statements were based mainly upon the changes in specific oxygen capacity and spectroscopic evidence. The spectroscopic evidence taken into consideration was mainly that the oxyhemoglobin modification showed an absorption band in the red at about the same position as that of methemoglobin and the two bands in the yellow and green of the oxyhemoglobin spectrum disappear as upon the addition of potassium ferricyanide. Fig. 7 shows the visible spectra of fresh oxyhemoglobin, oxyhemoglobin dialysate after 3 months dialysis, and methemoglobin,—all prepared from ox blood. The methemoglobin was prepared by the action of a few drops of saturated potassium ferricyanide solution on the oxyhemoglobin solution whose spectrum is shown. The dialysate of 0.12 per cent concentration is brown with a reddish cast. Its spectrum seems to be almost the same as that of methemoglobin of 0.08 per cent concentration. This is distinctly yellow. An absorption band in the red portion of the spectrum is seen in the spectroscope for both the oxyhemoglobin dialysate and the potassium ferricyanide methemoglobin. There is no discernible difference in position of this band, in the case of these two solutions. Fig. 7 shows the absence in the dialysate spectrum, as well as the methemoglobin spectrum, of the two bands, at approximately 578 and 545 μ , present in the oxyhemoglobin spectrum. The band beginning at about 398 μ on the spectrum of oxyhemoglobin, and also methemoglobin, appears to be identical on the dialysate spectrum.

This comparison leads to the conclusion that oxyhemoglobin in water solution is unstable and changes to a brown substance whose molecular aggregates under these conditions are of a size and character which allow them to pass through the collodion membrane. This brown modification of oxyhemoglobin is partly

methemoglobin, the fraction increasing upon long exposure to water and oxygen. The dialysate spectrum of Figs. 5 and 6 seems to be more like oxyhemoglobin and less like methemoglobin than in Fig. 7. The dialysate represented in Figs. 5 and 6 was prepared by dialysis during a shorter time than in the case of the material used for making Fig. 7. It would be interesting to compare this material with the brown modification obtained by drying oxyhemoglobin and discussed by Barcroft ((4) p. 60) and Van Slyke, Hastings, Heidelberger, and Neill (13).

Although the color and spectrum of the dialysate were different from the original oxyhemoglobin, it gave a positive precipitin reaction against the antiserum formed by injection of rabbits with the oxyhemoglobin.

Ox and hog carbon monoxide hemoglobin were dialyzed for 5 weeks under an atmosphere of CO gas. The solutions were protected from all but subdued light. This caution was considered necessary because, as Hartridge (14) has shown, carbon monoxide is split off by sunlight and the freed hemoglobin is susceptible to change to methemoglobin. This is irreversible; *i.e.*, methemoglobin does not combine with carbon monoxide. Carbon monoxide hemoglobin and oxyhemoglobin change rapidly to methemoglobin under the influence of ultra-violet light. Biuret and precipitin tests showed that no protein appeared in the dialysate. Evidently, then, none of the carbon monoxide hemoglobin had changed to the brown modification of oxyhemoglobin found in oxyhemoglobin dialysate.

SUMMARY.

1. The transmission curve of carbon monoxide hemoglobin spectrum is shown.

2. Evidence of the purity of the carbon monoxide hemoglobin, made by the method described, is presented in Fig. 2. No difference is indicated between spectra of uncrystallized and crystallized carbon monoxide hemoglobin made by this method.

3. Spectra of carbon monoxide hemoglobin from different species are shown to be alike.

4. A water solution of oxyhemoglobin is shown to change upon standing; in time methemoglobin is formed, probably with another modification of oxyhemoglobin. Carbon monoxide hemoglobin seems very stable in this respect.

The authors wish to thank Dr. William H. Welker for his valuable suggestions and advice during the course of the work.

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EXPLANATION OF PLATES.

PLATE 5.

FIG. 1. Approximate transmission curve of sheep carbon monoxide hemoglobin, 0.08 per cent (Kjeldahl).

FIG. 2. Horse carbon monoxide hemoglobin, crystallized and not crystallized, 0.08 per cent (Kjeldahl).

PLATE 6.

FIG. 3. Carbon monoxide hemoglobin, not crystallized, 0.08 per cent (Kjeldahl).

FIG. 4. Carbon monoxide hemoglobin, not crystallized, 0.08 per cent (Kjeldahl).

PLATE 7.

FIG. 5. Hog oxyhemoglobin and its dialysate, equal concentration (colorimetric).

FIG. 6. Hog oxyhemoglobin and its dialysate, equal concentration (Kjeldahl).

FIG. 7. Ox methemoglobin 0.08 per cent, oxyhemoglobin dialysate 0.12 per cent, and oxyhemoglobin 0.08 per cent (Kjeldahl).

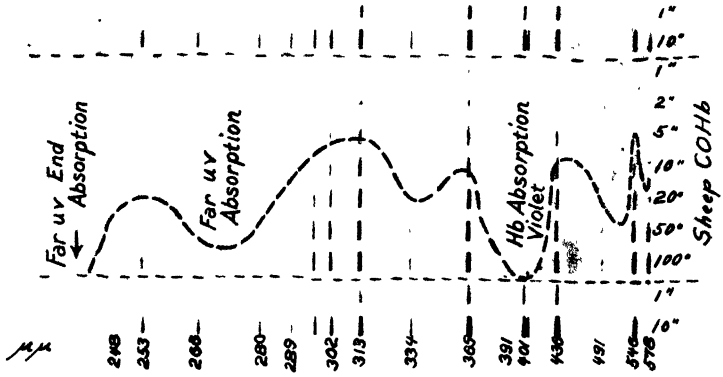


FIG. 1.

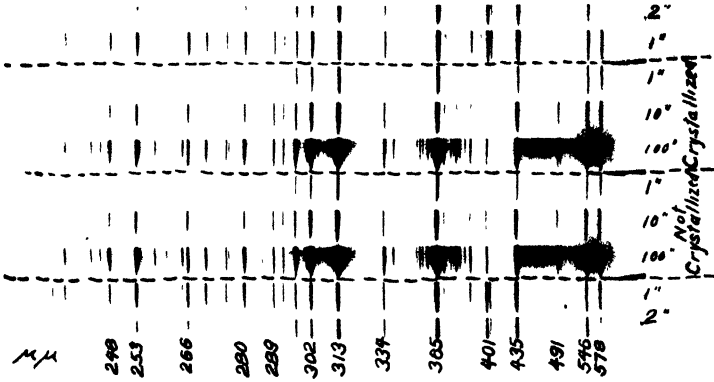


FIG. 2.

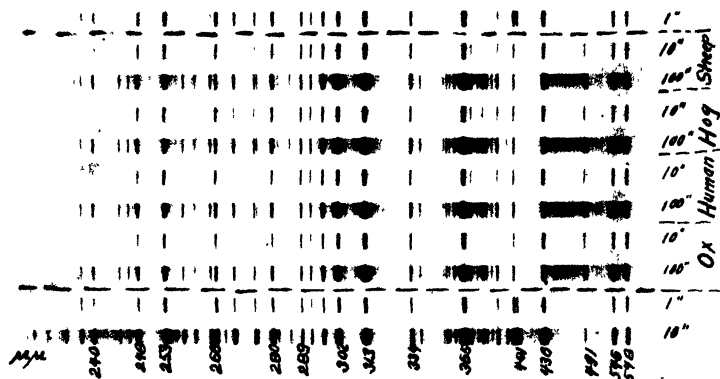


FIG. 3.

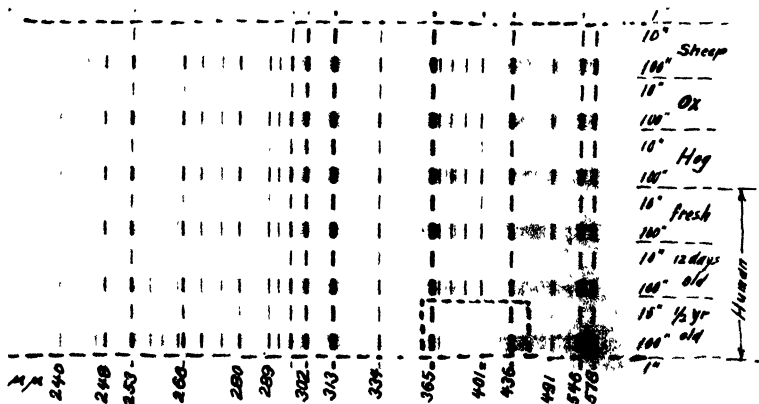


FIG. 4.

(Boor and Bachem: Carbon monoxide hemoglobin)

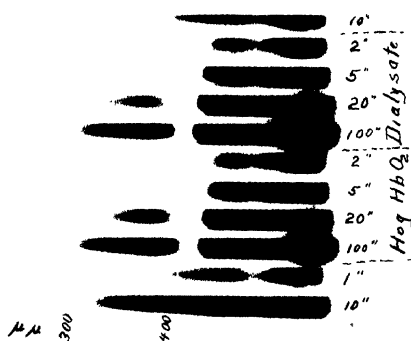


FIG. 5.

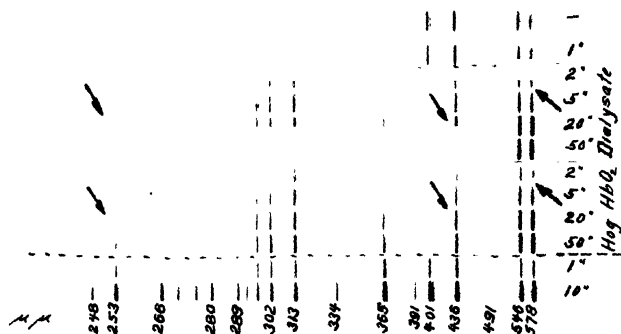


FIG. 6.

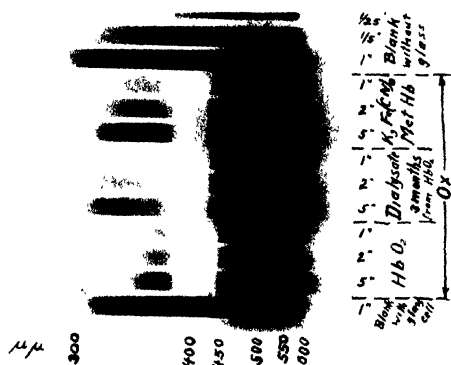


FIG. 7.

(Boor and Bachem: Carbon monoxide hemoglobin)

BENZOYLATED AMINO ACIDS IN THE ANIMAL ORGANISM.

V. THE SYNTHESIS OF GLYCINE AND OF HIPPURIC ACID IN RATS.*

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(Received for publication, November 18, 1929.)

Recently it was shown that the survival and growth of young rats on diets containing sodium benzoate were possible only if the diets furnished a supply of glycine, or a precursor of glycine, adequate for the detoxication of the benzoate and for the formation of new tissue proteins (1). In connection with these experiments, it was observed that certain toxic benzoate diets were less toxic if the rats were kept in cages resting on shavings rather than in raised cages. This fact suggested that glycine or some other dietary factor was obtained from the excreta or shavings. Subsequent work demonstrated that the original diets did not supply the optimum concentration of the vitamin B complex and that the ingestion of the excreta or shavings furnished the needed vitamins rather than glycine. The presence of additional vitamin B (B_1 and B_2) in these diets increased not only the tolerance of the rats for benzoate but also the rate of growth per gm. of food consumed. In this paper a study has been made of the effect of this increased efficiency of utilization of food on the synthesis of glycine and hippuric acid.

EXPERIMENTAL.

Male white rats, 4 to 5 weeks of age and weighing 45 to 60 gm. were used in these experiments. At the end of the experimental

* A preliminary report of this work was presented before the Society for Experimental Biology and Medicine, St. Louis, 1929 (Griffith, W. H., *Proc. Soc. Exp. Biol. and Med.*, **26**, 858 (1929)).

period of 40 days, an 18 to 24 hour urine sample was collected and analyzed for total benzoic acid (2), free benzoic acid (3), and hippuric acid (4). Analyses were always made on the combined urine of a group of rats on the same diet. The urine was preserved with 4 per cent sulfuric acid during collection.

The composition of the experimental diets is given in Table I. In every case Diet A was supplemented daily with 150 mg. of dried brewers' yeast,¹ corresponding to approximately 3 per cent of the diet. Diet B was similar to Diet A and contained 6 per cent of yeast. In most of these experiments the food intake was restricted to 210

TABLE I.
Composition of Diets.

Diet.....	A	3 A*	B	2.5 B	3 B	3.5 B	4 B	Calo- ries.
	gm	gm.	gm.	gm.	gm.	gm.	gm.	
Sodium benzoate.. . . .	0.0	3.0	0.0	2.5	3.0	3.5	4.0	
Casein†	35.0	35.0	35.0	35.0	35.0	35.0	35.0	112
Salt mixture‡.	4.0	4.0	4.0	4.0	4.0	4.0	4.0	
Sucrose.....	3.0	3.0	3.0	3.0	3.0	3.0	3.0	12
Cod liver oil.....	4.0	4.0	4.0	4.0	4.0	4.0	4.0	36
Corn-starch.....	36.4	31.0	28.6	24.1	23.2	22.3	21.4	304
Lard.....	17.6	20.0	19.4	21.4	21.8	22.2	22.6	
Yeast§.....			6.0	6.0	6.0	6.0	6.0	

* Throughout this paper the number before the letters A and B refers to the per cent of sodium benzoate in the diet.

† Commercial casein (12.75 per cent nitrogen). Calories were calculated on the basis that the casein was 80 per cent protein.

‡ Osborne and Mendel (5).

§ Dried brewers' yeast.

gm. during the 40 day period. This quantity of food was fed as follows: 4.5 gm. daily for the first 10 days, 5.0 gm. daily for the second 10 days, 5.5 gm. daily for the third 10 days, and 6.0 gm. daily for the last 10 days. When glycine was added to the diet, it replaced an equal weight of starch.

The results shown in Chart I demonstrated that Diet B was an adequate diet, at least for the 40 day experimental period. The food consumption and rate of growth on this diet were the same for

¹ We are indebted to Mr. O. F. Steidemann of the Laboratory Department of Anheuser-Busch, Inc., for the yeast used in these experiments.

the rats in raised cages as for the rats in cages on shavings. This rate of growth equaled that of control animals on the mixed stock diet. The restricted feeding experiments further demonstrated the adequacy of Diet B (Chart II). Because of the limitation of the food intake to 210 gm. during the 40 day period, these rats

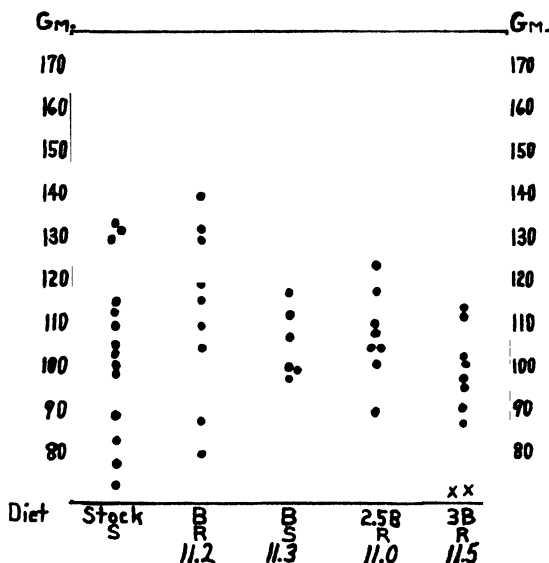


CHART I. Increase in weight of young male rats during a 40 day experimental period on control diets and on diets containing sodium benzoate. The per cent of sodium benzoate added to the basal diet, Diet B, is indicated by the number preceding the letter B. Diets were fed *ad libitum*. The letters R and S designate raised cages and cages on shavings respectively. Deaths during the experimental period are represented by X. The average calorific requirement per gm. increase in weight is indicated at the bottom of the chart. The food consumption may be obtained by multiplying the average calorific requirement per gm. by the average total increase in weight.

developed voracious appetites and it was reasonable to assume that those on shavings would have utilized any available nutrient material. However, the rats on shavings grew at the same rate as those in raised cages and it was apparent, therefore, that ingestion of shavings or excreta added no additional nutritive substances to the diet.

In experiments with Diet A the rate of growth of the rats in cages on shavings was greater than that of rats in raised cages and nearly equaled that obtained with Diet B (Chart II). Inasmuch as the same quantity of food of equivalent calorific value was ingested by the two groups of rats, it must be concluded that both the increase in the yeast supplement in Diet B and the access to

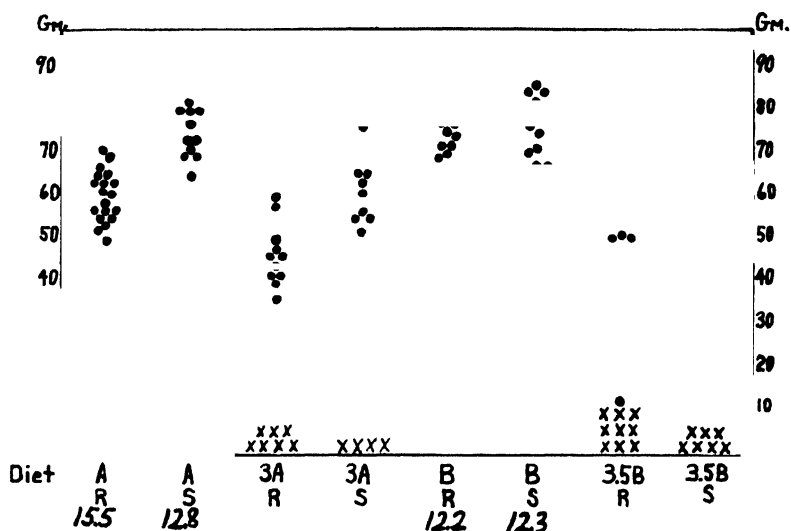


CHART II. Increase in weight of young male rats during a 40 day experimental period on control diets and on diets containing sodium benzoate. The per cent of sodium benzoate added to the basal diet is indicated by the number preceding the letter A or B. The food intake was restricted, each rat receiving the same quantity of food of equivalent calorific value (975 calories). The letters R and S designate raised cages and cages on shavings respectively. Deaths during the experimental period are represented by X. The average calorific requirement per gm. increase in weight is indicated at the bottom of the chart.

excreta and shavings in Diet A increased the efficiency of utilization of the food. Rats fed Diet A in raised cages required 15.5 calories per gm. increase in weight, whereas rats fed Diet A in cages on shavings required only 12.8 calories, and rats fed Diet B in raised cages required only 12.2 calories (Chart II).

Similar results were obtained with the toxic benzoate diets (Chart II). Better growth resulted in rats fed Diet 3 A in cages

on shavings than in raised cages. Such a difference was not found with Diet 3.5 B. Although this diet was definitely deficient in glycine, practically the same results were obtained on shavings as in raised cages. This demonstrated that neither glycine nor precursors of glycine were obtained from excreta or shavings.

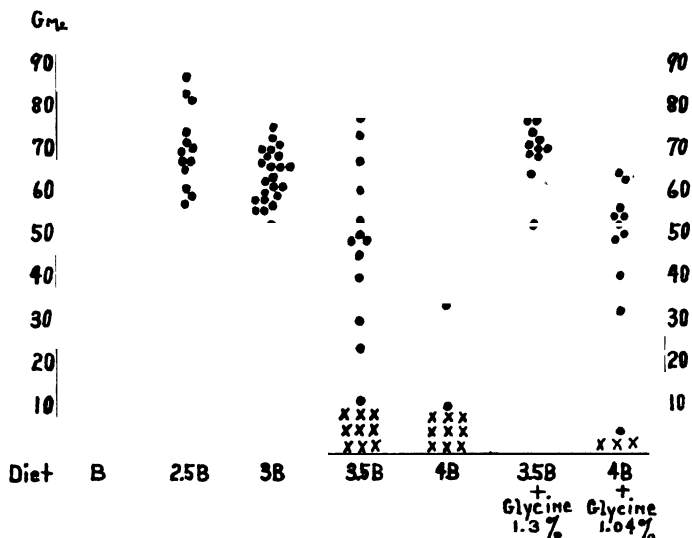


CHART III. Increase in weight of young male rats during a 40 day experimental period on diets containing sodium benzoate. The per cent of sodium benzoate added to the basal diet is indicated by the number preceding the letter B. The food intake was restricted, each rat receiving the same quantity of food of equivalent calorific value (975 calories). Rats were kept in raised cages. Deaths during the experimental period are represented by X. The detoxication of 1.0 gm. of sodium benzoate requires 0.52 gm. of glycine.

In a previous paper (1) it was shown that Diet A was not a satisfactory source of glycine, or of its precursors, if 3 per cent or more of sodium benzoate was present. With Diet 3 A seven out of twenty rats died during the experimental period and the rate of growth of the majority of the remainder was distinctly less than that of control rats on Diet A. Diet B proved a better source of glycine than Diet A. Good growth was obtained with Diet 2.5 B (Chart III). With Diet 3 B twenty-eight out of twenty-nine

rats survived, although the rate of growth was less than that of the controls on Diet B. The results with Diet 3.5 B closely resembled those obtained with Diet 3 A in the earlier experiments. With Diet 4 B only two out of eleven rats survived the 40 day period. The addition of 1.04 per cent of glycine to Diet 4 B greatly decreased the toxicity of this diet. The addition of 1.3 per cent of glycine to Diet 3.5 B resulted in satisfactory growth in eleven out of thirteen rats. In general the results with Diet B confirmed those previously obtained with Diet A, the two series differing only in the increased rate of growth and the slightly greater tolerance for benzoate of the rats on Diet B. The latter

TABLE II.

*Excretion of Free Benzoic Acid, Hippuric Acid, and Total Benzoic Acid on Diets Containing Sodium Benzoate.**

Diet.	No. of rats.	Total benzoic acid.	Hippuric acid calculated as benzoic acid.		Free benzoic acid.
		mg.	mg.	per cent of total benzoic acid	
2.5 B (Restricted).	7	626	582	93	39
3 B " 	15	785	685	87	53
3.5 B " 	10	692	538	78	64
3.5 B + 1.3 per cent glycine (restricted)	5	740	647	87	18

* Samples of urine were collected at the end of the 40 day experimental period.

point was illustrated by the results of the urine analyses. With Diet 3 A, 71 to 76 per cent of the ingested benzoate was excreted as hippuric acid (1), whereas with Diet 3 B 87 per cent was excreted as the detoxication product (Table II).

Rats fed Diet 2.5 B *ad libitum* showed the same rate of growth and consumption of food as control animals (Chart I). This was also true of ten out of twelve rats fed Diet 3 B *ad libitum*, two failing to survive the experimental period.

DISCUSSION:

The basal diet, Diet B, used in these experiments differed from that used previously, Diet A, in the amount of the yeast supple-

ment, Diet B containing twice as much yeast as Diet A. This change in the composition of the diet was associated with a marked increase (27 per cent) in the utilization of the food eaten. It was of particular interest, therefore, to determine whether those metabolic reactions which governed the formation of hippuric acid from benzoic acid and glycine, or precursors of glycine, were similarly affected. Although Diet 3 B was unquestionably less toxic than Diet 3 A, nevertheless it was not an adequate source of glycine. In both series of experiments the feeding of diets containing 2.5 per cent sodium benzoate resulted in growth comparable to that of control animals, while the feeding of diets containing 3 per cent benzoate resulted in decreased rates of growth. Certainly the ability to detoxicate benzoic acid by conjugation with glycine was not increased in these experiments to the same degree as was the utilization of the components of the diet for the formation of new tissues.

It is believed that the results of the feeding of such a diet as Diet 3 B may be used in estimating the ability of the rat to synthesize glycine. In these experiments, sodium benzoate was ingested by the rats in frequent small non-toxic doses and always in combination with foodstuffs. The total quantity administered was not in excess of that which could be detoxicated provided glycine was available. It was assumed, therefore, that in the rats fed Diet 3 B there was the maximum rate of detoxication of benzoic acid with glycine. During the 40 day period, 10.4 gm. of nitrogen were ingested and of this only 0.53 gm. appeared in the urine as glycine nitrogen and part of this presumably came from the pre-formed glycine of the diet. Less than 5.1 per cent of the total ingested nitrogen was excreted as glycine nitrogen under conditions which apparently were favorable for the synthesis of glycine. This result supported the conclusion of the previous paper (1) that the formation of glycine in rats from those nitrogenous substances normally giving rise to urea did not take place readily but rather was limited in extent. From one view-point the possible conversion of as much as 5 per cent of the protein nitrogen of the diet into glycine nitrogen represents synthesis of glycine on a relatively large scale. On the other hand, the formation of this amount of glycine was not at all comparable to the results of McCollum and Hoagland (6), for instance, who found that 37 per cent of the

total urinary nitrogen in the pig might be glycine nitrogen. Definite information regarding the synthesis of this amino acid in the rat, if synthesis really does occur, must await more exact determinations of the concentration of preformed glycine in casein and in the proteins of yeast.

SUMMARY.

1. The efficiency of the utilization of food in rats on diets containing sodium benzoate and deficient in the vitamin B complex was increased by permitting access to excreta and shavings or by increasing the supplement of yeast.

2. The more efficient utilization of food resulted in a marked acceleration of the rate of growth and in a less marked increase in the tolerance for sodium benzoate.

3. Under conditions favorable for the synthesis of needed glycine in rats less than 5 per cent of the total ingested nitrogen was excreted in the urine as benzoyl glycine.

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THE ABSENCE OF CALCIUM IN THE HUMAN RED BLOOD CELL.

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(Received for publication, December 7, 1929.)

Abderhalden (1), who studied the distribution of substances in the blood between plasma and red cells, reported the absence of calcium in the red corpuscles. He examined the blood of various animals such as the cow, bull, sheep, horse, pig, rabbit, dog, and cat and invariably found no calcium in the corpuscles. Marriott and Howland (2) studied the calcium of human blood. They accepted Abderhalden's findings as to the absence of calcium in corpuscles and did their studies on serum rather than on whole blood. Rothwell (3) studied the calcium content of human whole blood and obtained figures for calcium which agree with Abderhalden's assertion that there is no calcium in the red blood cells.

On the other hand Schmidt (4) claims to have found about 33 per cent of the total blood calcium in the corpuscles of human blood. Cowie and Calhoun (5) reported the presence of calcium in human whole blood in concentrations approximating that of serum. Jones (6), and Jones and Nye (7) reported figures ranging between 5.0 and 8.7 mg. of calcium per 100 cc. of corpuscles in the blood of infants and small children.

In view of these discrepancies a study was undertaken to reinvestigate this subject. Cowie and Calhoun, Jones, and Jones and Nye all used the nephelometric method of Lyman (8) for the determination of calcium. This method, however, was found by Clark (9) and by Denis and Minot (10) to give wide variations for the calcium content in blood. Clark found the error by this method to amount to 30 per cent.

EXPERIMENTAL.

We used essentially the method of Kramer and Tisdall (11), with some modifications in that the proteins were first removed with

trichloroacetic acid, and a weaker solution of permanganate was used for titration.

The blood was collected over powdered lithium citrate to prevent coagulation. A portion of the blood was centrifuged and the plasma removed to a clean tube. Hematocrit readings were taken in triplicate and the cell volume noted. Determinations were made on whole blood and plasma. The difference in calcium content between plasma and whole blood was taken to represent the calcium content of the corpuscles.

2 cc. of plasma or whole blood were diluted with 4 cc. of water and the proteins precipitated with 4 cc. of 20 per cent trichloroacetic acid. After standing for 10 minutes the mixture was filtered through ashless filter paper and 5 cc. (equivalent to 1 cc. of blood or plasma) of the filtrate placed in a centrifuge tube. To this was added 0.5 cc. of 4 per cent ammonium oxalate, and the trichloroacetic acid neutralized with ammonia water to pH 6.2 with brom-cresol purple as an indicator, as suggested by Shear and Kramer (12). A 20 per cent ammonia solution was used for the neutralization of the acid and 0.02 N solutions of ammonia and of hydrochloric acid for the adjustment of the pH. The mixture was left standing overnight. The calcium oxalate was then thrown down by centrifugation and washed twice with 2 per cent ammonia solution, the supernatant fluid being removed by decantation. 2 cc. of N H_2SO_4 were then added to the calcium oxalate; the tube was then placed in a beaker of boiling water for 5 minutes, and the oxalic acid titrated with 0.005 N KMnO_4 solution. By using this strength of permanganate no calculation was necessary, since the number of cc. of permanganate used in the titration multiplied by 100 gives the per cent of calcium directly. The permanganate solution was placed in a 2 cc. micro burette graduated to read to 0.001 cc. The tip of the burette was drawn out to a very fine capillary tube delivering about 100 drops per cc. During the titration a centrifuge tube similar to the one containing the oxalic acid, containing 3 cc. of water, was held alongside the oxalic acid tube. This served as a control to detect the faintest amount of color. The end-point was considered reached when the color did not fade after 1 minute.

This method was checked on serum against Clark's (9) modification of the Kramer-Tisdall method where the calcium is precipi-

tated directly in the serum in the presence of the serum proteins. The two methods checked closely, though somewhat higher figures were invariably obtained by the present method.

A large number of blood samples were thus examined. A small number of blood samples were examined for the calcium content in both plasma and serum; the results agreed fairly closely though, again, slightly higher figures were obtained for plasma.

The analyses showed, as judged by the amount of reduction of permanganate, the calcium content of the corpuscles to vary widely; in most cases there was a calcium content of about 30 per cent of the total blood calcium. Some blood samples showed a content of calcium in the corpuscles below 10 per cent, while in a few there was a larger amount of calcium in the corpuscles than in the plasma.

As the calcium content in blood serum is known to vary but slightly in different individuals, we questioned the accuracy of our results. Since a large number of reducing substances will reduce permanganate, we doubted that the reduction of permanganate was all due to the presence of calcium oxalate. Part of the precipitate obtained by treating with ammonium oxalate certainly did not behave as calcium oxalate in that it stuck rather tightly to the walls of the test-tube and did not dissolve in hot dilute sulfuric acid. However, it reduced permanganate. It seemed possible, therefore, that another substance, not calcium, which reduces permanganate, was carried down with the calcium oxalate. Rothwell (3) thought that this substance was blood protein carried down in the filtrate due to incomplete precipitation of the proteins with trichloroacetic acid. It may also possibly be due to peptides which are known to be carried down in the trichloroacetic acid filtrate.

Since, however, this substance, whatever its nature, is insoluble in hot dilute sulfuric acid, it was deemed advisable to substitute filtration of the calcium oxalate for centrifugation, thus leaving the foreign substance behind on the filter paper. Munktell No. 00 filter paper was used. This paper was found to retain all the calcium oxalate and allows rapid filtration.

The calcium was precipitated from the trichloroacetic acid filtrate as previously described with the following modification: Instead of using a centrifuge tube a test-tube was drawn out at the

bottom to a capillary about 2 inches long and sealed at the end. By holding this tube in a vertical position and adding the trichloroacetic acid filtrate carefully, the liquid, due to its surface tension, was drawn but slightly into the capillary, forming a closed pocket of air in the capillary, the solution resting on top of the inclosed air. Mixing of the reagents was accomplished by rotating the tube between the palms of the hands. After standing overnight the tube was clamped over a small funnel carrying a filter paper; the tube was closed with a stopper and the point of the capillary end of the tube broken off. The stopper was then slowly removed, allowing the contents of the tube to fall on the filter paper. Such a tube was found preferable to an ordinary test-tube in that it allows easier handling of the material and avoids the possibility of loss of material during the process of transferring the material to the filter paper and during washing the tube. The inside of the tube was then washed with a fine stream of 2 per cent ammonia water. The excess of ammonium oxalate was then removed from the filter paper by further washing the filter paper with small amounts of ammonia, spreading the ammonia in a fine stream all over the filter paper and letting it drain completely before adding new ammonia.

5 cc. of hot $\text{N H}_2\text{SO}_4$ were then poured slowly on the filter paper, small amounts at a time with complete draining before adding new acid. The filtrate was collected in a test-tube and titrated with 0.005 N permanganate solution as previously described. Duplicate checks were obtained by this procedure and added amounts of calcium were recovered from serum, plasma, and whole blood. A considerable number of determinations performed by this procedure showed definitely that no calcium is present in blood corpuscles (see Table I). The slight amount of calcium in the corpuscles shown in the table is considered to be due to experimental error.

In order to verify these findings further determinations were made on large volumes of human blood. Two samples of blood were obtained from patients with pulmonary edema, where withdrawal of blood was instituted as part of the treatment; 450 and 340 cc. samples of blood respectively were collected over powdered lithium oxalate from the two patients. The same general procedure was followed for the determination of calcium. A 1:5

dilution of the blood or plasma was made with trichloroacetic acid and filtered through filter paper. In order to obtain as much filtrate as possible the filter paper containing the trichloroacetic acid precipitate, after most of the filtrate drained through by gravity, was folded up and placed in a Buchner funnel; the mouth of the funnel was covered tightly with a piece of soft rubber and the rest of the filtrate was drained by applying suction. A measured amount of the filtrate was placed in a narrow, tall cylinder. The calcium was precipitated with 4 per cent ammonium oxalate,

TABLE I.
Distribution of Calcium in Plasma and Corpuscles.

Blood Sample No.	Cell volume.	Ca in whole blood.	Ca in plasma.	Ca in corpuscles.
	<i>per cent</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
1	37	6.8	10.8	0
2	38	7.3	11.3	0.3
3	38	6.8	11.0	0
4	34	6.8	10.2	0.1
5	36	6.7	10.7	0.1
6	39	6.8	11.0	0.2

TABLE II.
Absence of Calcium in Corpuscles.

Patient.	Plasma used.	0.02 N KMnO ₄ .	Ca	Whole blood.	0.02 N KMnO ₄ .	Cell volume.	Ca in corpuscles.
	<i>cc.</i>	<i>cc.</i>	<i>per cent</i>	<i>cc.</i>	<i>cc.</i>	<i>per cent</i>	
A	100	28.54	11.4	150	26.75	38	None.
B	75	19.69	10.5	100	16.62	37	"

with 0.5 cc. of oxalate for each cc. of blood or plasma, adjusted to the proper pH, 6.9, and after standing overnight was filtered and washed freely with ammonia water. Hot $\text{N H}_2\text{SO}_4$ was poured through the filter paper and the filtrate titrated with 0.02 N KMnO_4 . As Table II shows, no calcium was present in the corpuscles.

SUMMARY.

A study was made of the distribution of calcium between plasma and corpuscles in human blood. It was found, in confirmation of

Abderhalden's findings on blood of animals, that no calcium is present in the corpuscles of human blood.

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VARIATIONS IN BLOOD AND URINARY SUGAR AFTER THE INGESTION OF GALACTOSE.

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The experiments described in this communication show the hourly excretion of reducing substances into the urine following the ingestion of 20, 30, and 50 gm. of galactose by five normal men and two women. With the 50 gm. ingestion the behavior of blood-reducing substances was also followed hourly and for this part of the work seven normal men were studied in addition. We separated in both the urine and blood the fermentable and non-fermentable fractions of the reducing substance by means of yeast. Our primary object was to place on a quantitative basis, if possible, a series of observations by Rowe and his coworkers (1, 2) claiming a sex difference in the tolerance in women during various periods of their mature functioning life. The results we obtained, however, possess an interest of their own.

Analytical Methods.

Fermentable and non-fermentable sugar in urine and blood was determined by use of a modified Shaffer-Hartmann reagent (3).

I. Preparation from Urine or Blood of Solution Free from Interfering Substances.

(a) *Total Reducing Substances in Normal Urine.*—1 or 2 cc. of urine, depending on the concentration, are added to 2 cc. of 0.1 N H_2SO_4 , made up to 10 cc. with water, 0.3 gm. of Lloyd's reagent is added, and the mixture shaken and centrifuged. Interfering substances are thus removed by the technique of Folin and

Berglund (4) with smaller quantities of material. The supernatant liquid is used for "sugar" determination. With urine showing strong reducing power to Benedict's qualitative reagent, a previous dilution of 5 to 10 times is necessary before following the above routine.

(b) *Non-Fermentable Reducing Substances in Normal Urine.*—Fleischmann's yeast is washed according to the directions of Somogyi (5). The centrifuged material from the last clear washings is weighed, and water added to make a 25 per cent suspension. 1.25 cc. of the washed yeast suspension and 1 or 2 cc. of urine are mixed and warmed to 38° in a bath for 8 minutes with occasional agitation. 2 cc. of 0.1 N H_2SO_4 are added, the volume made to 10 cc. with water, 0.3 gm. of Lloyd's reagent added (5), the mixture shaken and centrifuged. The clear supernatant liquid is used for "sugar" determination. (In making the volume to 10 cc. with water the volume of the yeast suspension is reckoned as 1.0 cc.)

(c) *Total Reducing Substance in Blood.*—The Folin-Wu filtrate is used.

(d) *Non-Fermentable Reducing Substances in Blood.*—1 cc. of 25 per cent suspension of washed yeast is centrifuged, the water decanted, and any water adherent to the tube is removed by filter paper. The separated yeast is mixed with 6 cc. of Folin-Wu blood filtrate, warmed 8 minutes at 38° with occasional agitation, cooled, and centrifuged. This ferments glucose beyond a concentration of 250 mg. per 100 cc. of blood, without affecting galactose. It is with one slight variation the technique of Raymond and Blanco (6). The liquid after centrifuging is ready for "sugar" determinations.

II. "Sugar" Determination.

2.0 cc. of the Shaffer-Hartmann reagent, made without iodide, and 2.0 cc. of the liquid indicated in (a), (b), (c), or (d) are mixed in a 150 × 16 mm. tube, loosely stoppered, and heated 10 minutes in a rapidly boiling water bath. Cool to 30°. Add 2 cc. of 1 per cent KI solution and 2 cc. of 0.1 N H_2SO_4 . Titrate with 0.005 N $\text{Na}_2\text{S}_2\text{O}_3$. A control with 2.0 cc. of water instead of a solution containing sugar is made alongside the determinations on blood, and one with 2.0 cc. of water previously treated with H_2SO_4 and

Lloyd's reagent according to (a) alongside the determinations on urine.

Cc. thiosulfate for control — cc. thiosulfate for determination = cc. thiosulfate equivalent to mg. sugar

The reagent is standardized against pure sugars. It can be used for glucose, galactose, or lactose.

One of our reasons for adopting this particular technique was the reliability and simplicity of the modified Shaffer-Hartmann method in our hands for the determination of small amounts of reducing substance present in normal urine and in blood after

TABLE I.

Loss of Galactose in Fermentation of Urine by Large Amounts of Yeast. Subject in postabsorptive condition.

	Hr.	Urine volume.	Total sugar.	Non-fermentable "sugar."	
				20 gm. yeast.	1 cc. 25 per cent yeast suspension.
		cc.	mg.	mg.	mg.
500 cc. water at end of 0 hr.	0	25	24	21	20
	1	227	35	30	29
	2	296	22	19	17
	3	35	31	26	25
28.0 gm. recrystallized galactose in 400 cc. water at end of 0 hr.	0	32	30	21	25
	1	255	335	295	333
	2	410	61	49	59
	3	100	33	26	31

fermentation. Small increases due to ingested galactose are easily detected. Another reason in connection with the fermentation of glucose was the finding in our earlier experiments that with large amounts of yeast, as recommended by Folin and Svedberg (7) or by Somogyi (5), we experienced a loss of galactose. This was true either in water solution or in urine. A comparison of two series of urine samples, the first after the ingestion of 500 cc. of water, the second after the ingestion of 28 gm. of purified galactose in 400 cc. of water, and each analyzed for fermentable and non-fermentable "sugar" by large and small amounts of yeast, is

shown in Table I. The urine samples collected after water ingestion show the same amount of non-fermentable "sugar" with the varying amounts of yeast. Those after galactose ingestion, show a smaller amount of non-fermentable substance after the use of 2 gm. of washed yeast. This is especially true in the collection of the 1st hour when large amounts of galactose are excreted, and was a consistent finding. Had we not been cautious on this point we should have drawn the erroneous conclusion that during the excretion of galactose there occurred also a definite excretion of glucose. When the smaller amount of yeast is used, the difference between the fermentable and non-fermentable "sugar" becomes so small as to be within experimental error. At this point it might be noted that the subject used in the water ingestion experiment showed a very small though apparently definite amount of glucose in the urine in the fasting condition in all four samples of urine with either variation of yeast analysis. He thus showed a glycosuria in the sense expressed by Benedict, Osterberg, and Neuwirth (8). This was not present on every day, nor was it found in all other subjects. Our recoveries of added glucose to urine or blood by the methods thus described ranged from 98 to 101 per cent. Our recoveries of added galactose varied 95 to 100 per cent.

Experimental Conditions.

The subjects with three exceptions were normal laboratory workers or students. The exceptions were hospital patients in the surgical wards, whose condition and physical examination gave no reason to suspect any metabolic disturbance. These latter remained in bed during the test. No control of diet previous to the experiments was attempted. The following experimental routine was followed:

8.00 a.m. Empty bladder. Two cups weak tea, without sugar or cream; or 200 cc. water.

9.00 a.m. Urine Collection 0; blood Sample 0. x gm. of galactose in 200 cc. of water followed by 200 cc. of water.

10.00 a.m. Urine Collection 1; blood Sample 1.

11.00 a.m. Urine Collection 2; blood Sample 2.

12 noon. Urine Collection 3; blood Sample 3.

20, 30, or 50 gm. of galactose were given. Blood samples from an arm vein were taken with the ingestion of 50 gm. of galactose. Amounts of reducing substance in urine are reported as galactose, and in blood as glucose. We never found any fermentable substance in urine after galactose ingestion, beyond those very small amounts which might be normally and occasionally present in a few subjects and in view of lack of further evidence assumed the

TABLE II.

Constancy of Hourly Output of Non-Fermentable Substances in Urine after Ingestion of 500 Cc. of Water, Despite Variations in Urine Volume.

Date.	Urine volume per hr.	Non-fermentable "sugar" as galactose.
1929	cc.	mg.
Apr. 9	21	24
	24	26
	17	21
	17	23
Apr. 10	15	17
	57	20
	59	17
	39	18
Apr. 11	25	20
	227	29
	296	17
	35	25
Apr. 25	39	21
	98	19
	75	24
	40	17

extra reducing substance to be galactose. Mucic acid has been identified after nitric acid oxidation of urine following galactose ingestion, by Bauer (9) and by Wagner (10).

We satisfied ourselves that the ingestion of 500 cc. of water under the conditions of our experiments produced only slight variations in the output of non-fermentable reducing substance. Such slight variations as were noticed were quite independent of urinary volume, and the figures might be regarded as constant to ± 5

mg. (Table II). This approximate constancy enabled us to take the excretion of non-fermentable substance in Urine 0 as the standard and regard any excretion above the physiological variation as galactose arising from the sugar ingestion. It was for this reason that the urine results are reported as galactose. To illustrate the method of calculating and reporting the results of our experiments we show the details on subject Miss A. in Table III. In Tables IV and V, showing urinary excretion, the figures for hours 1, 2, and 3 only are reported.

We used different preparations of galactose at different times, including Eastman Kodak, Schuchardt's ordinary and Schuchardt's "puriss nach soxhlet." To test the influence of possible

TABLE III.

Results after Ingestion of 20 Gm. of Galactose at 9.00 A.M. by Subject Miss A., Showing Absence of Glucose and Excretion of Galactose into Urine.

Time.	Urine.		0.005 N thiosulfate.		Glucose	Non-fermentable "sugar."	Galactose
	No	Volume.	Total.*	After fermentation.†			
<i>a.m.</i>		<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
8.00- 9.00	0	30	0.73	0.72	None.	17	0
9.00-10.00	1	125	0.83	0.83	"	82	63
10.00-11.00	2	50	0.53	0.52	"	21	0
11.00-12.00	3	60	0.44	0.45	"	21	0

* See "Analytical Methods" I (a) and II.

† See "Analytical Methods" I (b) and II.

impurities on the results we carried out two experiments on the same individual, giving 30 gm. of Eastman Kodak galactose and 30 gm. of the same sugar after recrystallization from aqueous alcohol. The excretion of galactose was 223 and 307 mg. respectively.

Results.

Table IV shows the excretion of galactose into the urine in five men and two women after the ingestion of 20, 30, and 50 gm. of galactose. In those cases where two ingestions of the same amount of sugar were made the duplicate result is given. The duplicates show considerable variation in the amount of galactose excreted, but not so great that any confusion can arise as to the

general quantitative nature of the urinary response. Thus in subject F. H. v. N. the excretion of galactose after 50 gm. ingestion is 1072 and 1948 mg. in two experiments. This is almost 100 per cent variation but wide as that variation is, it cannot be confused with the response after the 30 gm. ingestion of galactose, which resulted in the excretion of 307 and 223 mg. (The hourly excretions in this latter experiment, resulting in the 223 mg. ex-

TABLE IV.

Hourly Excretion of Galactose after Ingestion of 20, 30, or 50 Gm. of Galactose.

Subject	T. H. N.			Miss A.				C. C. L.				F. H. v. N.			
Galactose ingested, gm.....	20	30	50	20	20	30	50	20	30	50	50	20	30	50	50
Urine No.	Galactose excreted, mg.														
1	27	132	125	36	63	141	139	71	212	360	278	37	277	828	1689
2	0	0	73	9	0	54	246	6	9	333	240	18	30	226	253
3	0	0	20	0	0	11	9	0	0	34	8	0	0	18	6
Total. . . .	27	132	218	45	63	206	394	77	221	727	526	55	307	1072	1948

Subject.....	V. J. H.				C. E. D.			Miss B.				Average.		
Galactose ingested, gm.....	20	30	50	50	20	30	50	20	30	50	50	20	30	50
Urine No.	Galactose excreted, mg.													
1	132	225	728	337	390	720	2202	302	812		373	132	360	706
2	13	47	913	907	34	106	1804	264	827	3495	2613	57	179	1003
3	0	0	47	46	6	0	94	0	0	531	270	0	2	90
Total. . . .	145	272	1688	1230	430	826	4100	566	1639	4026	3256	189	541	1799

cretion, are not given as the urine was collected in one sample of 3 hours.)

Table V shows the hourly excretion of galactose in all subjects after ingestion of 50 gm. of galactose and the blood "sugar" values as fermentable and non-fermentable fractions. It is to be inferred that the fermentable fraction represents glucose and any rise in the non-fermentable fraction represents galactose. In Table V the subjects are arranged in order of the total urinary galactose excretion. This arrangement brings out certain regularities.

Hourly Excretion of Galactose.—The appearance of galactose in the urine after its oral ingestion is prompt. It is always present the 1st hour and with the 20 and 30 gm. doses this represents the peak of excretion with one exception (Miss B.). The peak of urinary excretion is also at 1 hour with the 50 gm. dose in all but five experiments. The excretion has died away by the 3rd hour, and, in the few subjects tested, none is present in the 4th hour. With the 20 and 30 gm. doses, with two exceptions, the excretion is complete in 2 hours, and in these two exceptions the excretion in the 3rd hour is minimal. With the 50 gm. dose galactose is always present in the urine in the 3rd hour. With increased ingestion there is thus not only an increased excretion, but the point of maximal excretion is beginning to be shifted from the 1st towards the 2nd hour. This tendency to delay in excretion is seen in those individuals who excrete comparatively large amounts of galactose. Thus, with the 20 and 30 gm. doses the amounts excreted in the 2nd hour exhibit a general increase as we pass from those subjects showing a low total excretion to those showing a high total excretion, until we come to Miss B. who possesses a maximal excretion in the 2nd hour. This behavior of the individual reaction and effect of increasing dose coincides well with those results in the literature, where subjects taking 100 gm. of galactose (i.e., over 1 gm. per kilo of body weight) have shown prolonged excretion. In this connection it is interesting to note that Miss B's weight was only 43 kilos. Body weight, however, is not the only factor. The results in Tables IV and V are by no means in the same order as the body weights of the individuals.

Total Excretion of Galactose.—Before commencing this work, from a general reading of the literature on galactose tolerance, there was in the minds of the authors an impression that galactose was a poorly utilized sugar. This impression is brought about by the continual occurrence of phrases such as "galactose is a poor glycogen former;" "galactose is only slowly converted into glucose;" "the tolerance for galactose is low;" "whereas normal individuals tolerate 100 gm. of glucose, urinary sugar is present after 20 to 30 gm. of galactose." Such an impression is not borne out by our experiments, nor is it always a correct conclusion from other experimental data in the literature (1, 2, 4). From Table IV it is evident that all the subjects showed galactosuria

after a 20 gm. ingestion of galactose. It seems probable that galactose would still have been found in the urine had we used 10 gm. as the minimal dose. Indeed Rowe (1) shows instances where a qualitative Benedict test for sugar is given by the urine after 10 to 20 gm. of galactose. Folin and Berglund (4) observed urinary galactose after a 10 gm. ingestion in two subjects. From a qualitative standpoint the utilization of galactose would appear to be poor. From a quantitative standpoint, however, the utilization of galactose wears a different aspect. The amounts secreted vary greatly with different individuals but the average loss is not as high as 10 per cent. Our most extreme case is subject W., as shown in Table V, who excreted nearly 7 gm., a utilization of 86 per cent. On the same intake half our subjects show a utilization of over 97 per cent. The subjects of Folin and Berglund show a utilization of the same magnitude on small amounts of galactose ingestion. The normal subjects cited by Rowe (1) show a utilization of approximately 97 per cent. Cori (11) under conditions of maximum absorption of galactose in rats found, however, a 60 per cent excretion in 4 hours.

Against the possible objection that our figures represent only a part of the excretion of galactose and that part of the remainder is stored only to be slowly released and excreted at a later period it may be pointed out that both the blood sugar curves and the urine curves show return to normal figures in 3 hours. There is no evidence of the absorption and slow excretion of galactose such as is observed in the case of non-fermentable carbohydrate products obtained from ordinary foodstuffs as noted by Folin and Berglund. Our observations harmonize with the idea that following a single dose of galactose a small percentage escapes utilization during the period of maximal absorption. The remainder is conveyed to the tissues and organs and either stored or immediately utilized. How rapid can be its utilization and storage can be inferred from the study of the blood sugar curves. Why a small amount escapes can be correlated with the absence of a demonstrable renal threshold for galactose.

Non-Fermentable "Sugar" in Blood.—This fraction of the blood sugar shows little or no alteration in the subjects composing roughly the first half of Table V. Of the first eight subjects only subject L. shows a marked rise. Subjects J. S., C. C. L., and

possibly V. J. H. show sufficient variation to be characterized as due to the galactose ingestion, when it is noticed that the peak of the non-fermentable fraction of the blood sugar (small as it is) corresponds with the peak of the galactose excretion in the urine. The latter part of Table V shows unmistakable increases in the non-fermentable fraction of the blood sugar. In the last three subjects the increases are to be observed in the 2nd hour specimen as well as the 1st. It would appear a simple conclusion that this

TABLE V.

Urinary Excretion of Galactose and Hourly Blood Sugar after Ingestion of 50 Gm. of Galactose.

Subject.	Galactose excreted, mg.				Hourly blood sugar (mg. per 100 cc.)							
	1st hr.	2nd hr.	3rd hr.	Total	Fermentable.				Non-fermentable.			
					0 hr.	1st hr.	2nd hr.	3rd hr.	0 hr.	1st hr.	2nd hr.	3rd hr.
C. H. D.	101	39	41	181	87	86	87	81	37	37	36	34
T. H. N.	125	73	20	218	78	77	77	78	42	43	43	43
J. S.	101	171	23	295	82	81	89	70	45	45	52	46
Miss A.	139	246	9	394	75	86	75	72	39	39	39	39
C. C. L.	360	333	34	727	81	106	75	65	38	44	38	34
V. J. H.	337	907	46	1390	75	128	80	73	42	35	47	38
L.	916	497	10	1423	80	85	73	79	42	120	49	43
F. H. v. N.	1689	253	6	1948	85	105	76	79	43	43	42	42
L. F.	68	1656	365	2089	71	85	91	81	37	48	88	39
G. S.	1064	968	115	2133	91	98	96	91	32	86	42	33
A.	1777	1213	66	3056	88	87	77	77	31	99	33	33
Miss B.		3495	531	4026	87	91	90	85	40	133	75	42
C. E. D.	2202	1804	94	4100	78	76	78	81	42	144	61	39
W.	2907	3689	224	6820	67	79	76	72	47	187	95	49

gradation in the amount of the non-fermentable fraction of blood sugar observable after galactose ingestion is dependent on the rate of absorption of the sugar from the alimentary tract. Such subjects as compose the first part of Table V absorb galactose relatively slowly, so that little excess finds its way into the urine. With a more rapid absorption the galactose accumulates in the blood for a short period, and also shows a marked increase in the urinary excretion.

Folin and Berglund observing the excretion of galactose after

such small doses as 10 gm. by mouth postulated the absence of a renal threshold for galactose. Such a postulate is very probable. Subjects C. H. D., T. H. N., Miss A., and F. H. v. N., though showing unmistakable amounts of urinary galactose, showed no alteration in the non-fermentable blood sugar fraction. It is possible, of course, that our method of hourly observations was at fault and that determinations made at more frequent intervals would have revealed increases in this fraction. In the two latter subjects just mentioned, however, there were present in the urine considerable amounts of galactose in both the 1st and 2nd hour collections. Had there been the necessity for a rise to a definite level of blood galactose before its occurrence in the urine, it should certainly have been observable in the blood specimen taken 1 hour after the sugar ingestion.

At this point we must draw attention to what appears to be a discrepancy between our values for the non-fermentable blood "sugar" and those of other observers. Our figures range from 31 to 47 mg. per 100 cc. in the fasting blood and expressed as glucose, giving an average of nearly 40 mg. This is much higher than the figures of other observers. It is also much higher than a series of values obtained by the same method in this laboratory when the range was found to be 15 to 32 mg. with an average of 26 mg. (3). It is evident that the results are consistent among themselves and do not affect the conclusions we have drawn regarding the presence or absence of galactose in the blood during our experiments. Recent experiments have emphasized the variability of the values for blood sugar as obtained by different analytical methods. The results given in this paper were all obtained with one large preparation of the modified Shaffer-Hartmann reagent. The values for blood glucose agree with those of other observers and our values for pure glucose and galactose agreed with values obtained by previous preparations of this reagent.¹

Fermentable Sugar in Blood.—As well as alterations in the non-fermentable fraction of blood sugar we have found alterations in the glucose. In four subjects (C. C. L., V. J. H., F. H. v. N., and L. F.) it is most striking and beyond any possibility of experimental error. Increases of smaller magnitudes are also observable in

¹ A new preparation of the reagent has given the usual range of values for "non-fermentable sugar" in blood.

five other subjects. Of these nine, the blood glucose peak coincides with the urinary galactose peak in six subjects and with the blood galactose peak in six subjects, two other subjects showing no alteration in the non-fermentable values.

The finding of a rise in blood glucose is at variance with the results after the administration of galactose by Corley (12) to the rabbit and by Cori and Cori (13) to the rat. Blanco (14), however, noticed no increase in blood galactose, but an increase in blood glucose after oral administration of galactose to rabbits, non-fasting, and under amytal anesthesia. The variability of our own results, however, precludes the drawing of any too far reaching generalization. There are evidently differences depending upon species, individuals, doses, and conditions. Further progress will depend on the more exact limitation of some of these factors. Provisionally, however, it is possible to draw the conclusion that the conversion of galactose to glucose can be much more rapid than has hitherto been supposed.

We have not considered as serious an argument that the rise in blood glucose in the four subjects mentioned above is an emotional hyperglycemia. Both V. J. H. and F. H. v. N. are well accustomed to metabolism experiments and the taking of blood specimens and have shown normal and expected values under other conditions. Subject C. C. L., however, was very nervous previous to the taking of the initial blood specimen. The blood glucose, however, was normal. His apparent nervousness had vanished at the time of the taking of the later specimens. Subject L. F. behaved stolidly throughout the experiment.

On the supposition that galactose is rapidly converted to glucose it is perhaps not without significance that those of our subjects who show the largest increase in blood glucose are those occupying the intermediate positions in Table V. The extreme variations in blood sugar and urinary sugar changes become more understandable if we assume variations in the absorption rate and variations in the conversion rate of galactose, as well as the ordinary sugar-removing mechanisms. Those subjects at the beginning of Table V can be represented as possessing relatively slow absorption rates, accompanied by sufficiently high conversion and removal rates to maintain a nearly unchanged blood equilibrium. A small amount of galactose, however, escapes into the urine.

With a more rapid absorption there can be an accumulation of glucose in the blood, if the rate of conversion of galactose to glucose is sufficiently rapid. Such perhaps are our four subjects, C. C. L., V. J. H., F. H. v. N., and L. F. Without a sufficiently rapid conversion of one sugar to the other, the amount of galactose in the blood becomes extremely high, masking any slight increase in blood glucose and giving rise to a high urinary output of galactose.

On such a theory it would appear to be of advantage to use moderate or small doses of galactose to be able to observe the complete series of changes, and in this connection some observations of Rowe and Chandler may be of interest. These authors observed the blood and urinary changes after the ingestion of amounts of galactose too small to cause a melituria as judged by Benedict's qualitative reagent. Some of their subjects showed marked changes in total blood sugar values. Had the increase in blood sugar been due to galactose, it seems very improbable that there would not have been in the urine sufficient sugar to have given a positive test with the Benedict reagent. Certainly no subject of ours showing a like increase in blood galactose failed to show a marked urinary response, and the authors themselves are evidently at a loss to explain the lack of connection between blood and urine sugar findings, for they emphasize the lack of correlation. On the same evidence they are doubtful of the lack of a renal threshold for galactose and are inclined to attribute the results of other observers to the presence of impurities. On the supposition that the rises in blood sugar observed by these authors were due mainly to glucose and not to galactose the results become more intelligible.

Quite apart from any theory of conversion of galactose to glucose the total blood sugar curve of subject L. F. is of interest. Among other observers, Foster (15) has noted blood sugar curves after the ingestion of galactose which continued to rise for some 3 or 4 hours. The blood sugar curve of L. F. continues to rise for 2 hours and is the only one failing to return to the initial level. It appears intermediate in character between the majority of our curves and those obtained by Foster.

SUMMARY.

The hourly excretion of galactose after ingestion of 20, 30, and 50 gm. of galactose has been noted in fourteen normal subjects.

There is always a small amount of galactose in the urine after galactose ingestion.

The total amount of galactose excreted varies greatly with different individuals.

The simultaneous behavior of the blood sugar has also been studied after the ingestion of 50 gm. of galactose.

Individuals showing only a small excretion of galactose show little change in blood sugar values.

Figures are given confirming the probable absence of a renal threshold for galactose.

Individuals showing moderate or large amounts of galactose in the urine usually show a marked increase in the non-fermentable function of blood "sugar."

Marked increases in blood glucose have also been noted in some individuals though the only sugar found in the urine has been non-fermentable.

It is suggested that the conversion of galactose to glucose can be much more rapid than has previously been supposed.

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DO THE SPECTROGRAMS OF KAHLENBERG AND CLOSS DEMONSTRATE THE PRESENCE OF ALUMINUM IN BIOLOGICAL MATTER?

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(Received for publication, November 15, 1929.)

Our paper "A Study of the Possible Rôle of Aluminum Compounds in Animal and Plant Physiology,"¹ has been the subject of two communications² by Kahlenberg and Closs. They contend on the basis of spectrographic experiments that we are in error in asserting that, "Aluminum is not a constituent of either plant or animal matter." Our views concerning the possible content of aluminum in plant and animal tissues are more accurately expressed in the text of our paper, where we interpret our results as "indicating the absence of aluminum down to less than 0.5 p.p.m. of the fresh tissue." We have made no positive assertions as to the absence of aluminum from such substances beyond the approximate limits of the technique which we employed in testing for this element.

In support of their criticisms of our work Kahlenberg and Closs submit experimental data which consist exclusively of spectrograms of the ashes of a number of plant and animal products. In the preparation of these spectrograms Kahlenberg and Closs have not followed the customary procedure in using a Hartman diaphragm of introducing into the third spectrum, lines of the substance tested for, in this case aluminum, a procedure which greatly facilitates the identification of lines of the element tested

¹ McCollum, E. V., Rask, O. S., and Becker, J. E., *J. Biol. Chem.*, **77**, 753 (1928).

² Kahlenberg, L., and Closs, J. O., *Science*, **69**, 186 (1929); *J. Biol. Chem.*, **83**, 261 (1929).

for. Nevertheless, they contend that all of these spectrograms show aluminum lines. We have examined the published illustrations of their spectrograms and can find no evidence in any of them of lines 3961.5 and 3944.0, the *raies ultimes* of aluminum. Their spectrograms contain only two lines in the 3900 region, but those must be 3968.5 and 3933.7, the *raies ultimes* of calcium, since calcium is a constituent of all substances which they represent by spectrograms. Apparently, therefore, Kahlenberg and Closs have mistaken lines 3968.5 and 3933.7, the *raies ultimes* of calcium, for lines 3961.5 and 3944.0, the *raies ultimes* of aluminum. Their spectrograms would have contained all of the above four lines if their samples had contained aluminum. The above calcium lines are almost invariably prominent and conspicuous. The aluminum lines, if present, would have occurred between these calcium lines. If the lines they refer to aluminum are to be assigned to this element, *where are the calcium lines; viz.*, 3968.5 and 3933.7.

Furthermore, it is significant that their spectrograms do not contain any other persistent lines of aluminum, such as 3092.7 and 3082.2, which are not subject to confusion with calcium lines.

We were aware of the possibility that the original negatives of Kahlenberg and Closs might have contained aluminum lines so faint that they could not be reproduced in the published illustrations, and therefore requested of Dr. Kahlenberg an opportunity to examine his negatives. He was unwilling to allow these to go out of his files and since it has not been possible for us to visit his laboratory for this purpose we requested prints from these negatives upon which the lines assumed to indicate aluminum were marked. Dr. Kahlenberg replied to this request that pressure of work prevented the preparation of such prints, but assured us "that the reproductions in the J.B.C. are good ones." His assurance on this point warrants our belief that aluminum lines are not present on his negatives and warrants our conclusion that he has mistaken calcium lines for aluminum lines.

In further correspondence Dr. Kahlenberg declared "that the lines in question have been checked with the lines obtained from pure aluminum compounds, and there is consequently no reason to believe that there is any confusion as to their identity." However, in this case such an experiment is no check or control unless or until after it has been supported by other and similar experi-

ments with pure calcium compounds showing that the lines produced by calcium do not coincide with the lines in question. Only such a procedure would demonstrate whether or not the lines under consideration are produced by calcium in the absence of controls consisting of the biological ashes to which small quantities of aluminum had been added after the test spectrum had been photographed. The control spectrum, *i.e.* the spectrum of the biological ash containing the added aluminum, should have been placed directly underneath the test spectrum by means of the third aperture in the Hartman diaphragm.

PRESENCE OF ALUMINUM IN ANIMAL AND PLANT MATTER.

By LOUIS KAHLENBERG AND JOHN O. CLOSS.

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(Received for publication, November 30, 1929.)

Replying to the accompanying article of McCollum, Rask, and Becker, we wish to state that the spectral lines in question, namely 3961.5 and 3944.0 Å., have been carefully checked with the lines obtained from pure aluminum oxide, aluminum chloride, and aluminum sulfate and there is consequently no doubt as to their identity. In other words, confusion with calcium or any other lines does not exist. Dr. McCollum was informed to this effect by letter on October 7. His attention has also been called to the articles of F. P. Underhill and coworkers in the September number of the *American Journal of Physiology*, who have even made quantitative estimations of aluminum in organic material by chemical means. Such results show that aluminum exists in plant and animal matter in far greater quantity than spectroscopic traces, so that in the spectroscopic detection of aluminum in plant and animal ash one ought, at least in some cases, to find it possible to get a fairly complete spectrum of aluminum. In other words, one ought not to be entirely dependent upon the prominent lines 3961.5 and 3944.0 Å., which lie close to calcium lines, but to find some of the other less prominent yet quite characteristic aluminum lines substantiating further the presence of that element. Among such lines are the pairs 3092.7-3082.2, 2660.5-2652.6, and 2575.2-2568.1 Å. To bring out any of these less prominent pairs (especially when aluminum is present in tiny amounts) requires special manipulation as to preparation of the material to be tested, adjustment of the arc, time of exposure, time of development, and even sensitiveness of the particular films or plates used. (Compare in this connection the discussion

by Kayser in his recent book.¹) Work in this direction is in progress in this laboratory; and doubtless these doublets can be brought out by very careful attention to details of manipulation. However, these labors would hardly be so necessary now since aluminum is actually being estimated quantitatively chemically in organic materials, and since McCollum himself interprets his results merely as "indicating the absence of aluminum down to less than 0.5 p.p.m. of the fresh tissue."² This is quite different from his general conclusion, "Aluminum is not a constituent of either plant or animal matter."³

¹ Kayser, H., *Tabelle der Hauptlinien der Linienspektren aller Elemente, nach Wellenlänge geordnet*, Berlin, p. v (1926).

² It is interesting to compare the quantitative estimations of Underhill and coworkers in this connection (Underhill, F. P., and Peterman, F. I., *Am. J. Physiol.*, **90**, 15, 40, 62, 67, 72 (1929)).

³ McCollum, E. V., Rask, O. S., and Becker, J. E., *J. Biol. Chem.*, **77**, 767 (1928).

ON THE CARBOHYDRATE OF THYMONUCLEIC ACID.

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(Received for publication, January 9, 1930.)

In an article by Levene and Mori,¹ the properties of the desoxypentose obtained from thymonucleic acid were compared with those of the synthetic *d*-xylodese and the synthetic ribodese. On the basis of this comparison, the identity of the natural sugar with xylodese was excluded. However, the natural desoxypentose differed also from the synthetic ribodese. The possibility was then considered that the synthetic sugar was a mixture of the *d* and *l* forms, and that the partial change of the *l* form into the *d* form was the result of the chemical reactions involved in the process of preparation. We therefore repeated the synthesis of ribodese under exactly the conditions described by Levene and Mori and we found that the synthetic *l*-ribodese and its benzylphenylhydrazone showed rotations of exactly the same value as the desoxypentose of thymonucleic acid and its benzylphenylhydrazone respectively, the only difference being the direction of rotation. The melting points are identical. Hence, the carbohydrate of thymonucleic acid is *d*-2-ribodese.

We also took this occasion to prepare the two desoxypentonic acids. However, in view of the other findings, only a small quantity of the acid from the natural sugar was prepared and its rotation was therefore not determined.

It is difficult at this date to ascertain the cause for the findings of last year. Reexamination of the several previous samples of ribodese confirmed the rotations then recorded. One sample, containing traces of barium and for that reason not examined last year, has an initial rotation of $[\alpha]_D^{25} = +36^\circ$ and at equilibrium

¹ Levene, P. A., and Mori, T., *J. Biol. Chem.*, **83**, 803 (1929).

$[\alpha]_D^{25} = +20.81^\circ$. Of two remaining samples of the old arabinal, one has a rotation of $[\alpha]_D^{25} = -80^\circ$ and the other $[\alpha]_D^{25} = -166^\circ$. The rotation recorded for this substance by Meisenheimer and Jung² is $[\alpha]_D^{19} = -202.8^\circ$ and by Gehrke and Aichner³ is $[\alpha]_D^{20} = -100.9^\circ$. Last year the rotation of our best preparation was $[\alpha]_D^{25} = -199^\circ$. The possibility is not excluded that some of the *l*-arabinose then used was by error mixed with *d*-arabinose.

EXPERIMENTAL.

β -l,2-Ribodeseose.—This sugar was prepared exactly as described by Levene and Mori¹ from an arabinal with a rotation of $[\alpha]_D^{25} = -263.55^\circ$ (in CHCl_3). The optical rotations of the analytically pure substance were as follows:

Immediately after dissolving in pyridine,

$$[\alpha]_D^{25} = \frac{+3.17^\circ \times 100}{1 \times 3.456} = +91.7^\circ$$

and at equilibrium,

$$[\alpha]_D^{25} = \frac{+1.49^\circ \times 100}{1 \times 3.456} = +40.5^\circ.$$

It began to soften at 67° , melted at 80° , and clarified completely at 154° .

4.365 mg. substance: 7.190 mg. CO_2 and 3.025 mg. H_2O .

$\text{C}_8\text{H}_{10}\text{O}_4$. Calculated. C 44.75, H 7.51.

Found. " 44.91, " 7.75.

0.2 gm. of the sugar was converted into the benzylphenylhydrazone as described by Levene and Mori. The hydrazone melted at 125 – 126° (uncorrected) and showed a rotation of

$$[\alpha]_D^{25} = \frac{+0.35^\circ \times 100}{2 \times 1.060} = +17.5^\circ \text{ (in pyridine).}$$

6.780 mg. substance: 0.524 cc. N at 26° and 766.5 mm.

$\text{C}_{18}\text{H}_{22}\text{O}_3\text{N}_2$. Calculated. N 8.91.

Found. " 8.89.

² Meisenheimer, J., and Jung, H., *Ber. chem. Ges.*, **60**, 1462 (1927).

³ Gehrke, M., and Aichner, F. X., *Ber. chem. Ges.*, **60**, 918 (1927).

3.27 gm. of the sugar were oxidized by the method described by Goebel,⁴ with slight modifications. The sugar was dissolved in the iodine solution and cooled to -5° before the barium hydroxide solution was added. The latter was added in the course of 5 minutes. The mixture was then stirred for 5 minutes more before the lead carbonate and sulfuric acid were added. The acid was then converted into the barium salt as described by Goebel. In order to purify the salt it was dissolved in a small amount of water and reprecipitated with a mixture of alcohol and ether. It was obtained as an amorphous snow-white powder.

The rotatory power of the salt and free acid were determined as follows: 0.8126 gm. of salt was dissolved in water and diluted to 5 cc. Rotation in a 2 dm. tube was found to be -0.14° .

$$[\alpha]_D^{25} = \frac{-0.14^{\circ} \times 100}{2 \times 16.252} = -0.43^{\circ}.$$

0.5 cc. of 36 per cent hydrochloric acid was then added to the 5 cc. of solution in the polariscope tube. Immediately after mixing the reading was $\alpha = +1.74^{\circ}$. Calculated on the basis of the free acid

$$[\alpha]_D^{25} = \frac{+1.74^{\circ} \times 100}{2 \times 10.18} = +8.5^{\circ}.$$

12 hours later the reading was $\alpha = -2.18^{\circ}$. Hence for lactone

$$[\alpha]_D^{25} = \frac{-2.18^{\circ} \times 100}{2 \times 8.96} = -12.2^{\circ}.$$

The salt analyzed as follows:

5.990 mg. substance: 6.100 mg. CO_2 and 2.155 mg. H_2O .
9.910 " " : 5.330 " BaSO_4 .

The salt obtained from the natural sugar analyzed as follows:

7.000 mg. substance: 7.040 mg. CO_2 and 2.515 mg. H_2O .
6.960 " " : 3.725 " BaSO_4 .

$\text{C}_{10}\text{H}_{18}\text{O}_{10}\text{Ba}$.	Calculated.	C 27.56, H 4.13, Ba 31.55.
	Found.	" 27.77, " 4.02, " 31.65 synthetic.
		" 27.42, " 4.02, " 31.49 natural.

⁴ Goebel, W. F., *J. Biol. Chem.*, **72**, 802 (1927).

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